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THE ANTIGENIC FUNCTION OF SIMPLE CHEMICAL COMPOUNDS: PRODUCTION OF PRECIPITINS IN RABBITS.

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IT has long been known that certain simple chemical compounds are capable of producing allergic phenomena in man, and that many of these substances may also be used experimentally to induce skin sensitization in guinea-pigs. It is now generally accepted, largely as the result of the work of Landsteiner and his colleagues (1945), that such phenomena have an immunological basis; nevertheless the precise nature of the antibodies involved remains in doubt, and there seems to be a demarcation between the antibody response to the application of a chemical sensitizing agent and the response to parenteral injection of a full protein antigen.

This differentiation appears particularly clearly from the work of Landsteiner. In general, intradermal injection of a strong sensitizing agent, such as picryl chloride, leads to skin sensitization and (in the guinea-pig) usually to the anaphylactic state, but not to the production of precipitating antibodies; intraperitoneal injection of the sensitizer does not in the ordinary way lead to skin sensitivity if precautions are taken to avoid contact with the skin. On the other hand, intraperitoneal injection of an antigen consisting of a conjugate of the sensitizer with protein gives rise to specific precipitin formation but not to sensitization. The same picture has emerged clearly from the work of one of us (Gell, 1945) on sensitization to Tetryl; in this case a special study was made of the relation of circulating antibodies to skin sensitivity, and it was found that the presence of precipitating antibodies in the blood specifically directed against the sensitizing group had no effect whatever in enhancing or diminishing the sensitivity produced by intradermal injection of the sensitizer.

Some progress towards bridging the gap between the two types of antibody response has been made by Landsteiner and Chase (1940, 1941) in experiments with picryl chloride; as has already been mentioned, intradermal injection of this compound leads, in the guinea-pig, to skin sensitization and the anaphylactic state; moreover, the presence of anaphylactic antibodies in the blood can be demonstrated by passive sensitization of normal animals. Although the

intraperitoneal injection of picryl chloride by itself does not produce such effects, sensitization can be induced by intraperitoneal injection of picryl chloride with killed tubercle bacilli as an adjuvant, or, less effectively, by injection of " picrylated " red cell stromata. In both these cases the immunizing preparation is such as to cause a local foreign body reaction, and it has been suggested that the special type of sensitizing and anaphylactic antibody is carried in the characteristic cells of the exudate. Indeed Landsteiner and Chase (1942) claim to have transferred skin sensitivity passively by injecting a normal animal with the centrifuged deposit of such an exudate.

We have failed to find records of attempts to bridge the gap from the other direction, i.e. by the observation of circulating precipitins after sensitization by the intradermal route, except for the passing reference by Landsteiner and Chase (1937) to the occasional occurrence of precipitins in guinea-pigs sensitized to picryl chloride ; in spite of the success of sensitization through the intraperitoneal route by the special techniques just referred to, the original distinction drawn between the actions of chemical sensitizers and full protein antigens seems therefore to hold good. This being so it is of interest to inquire whether there is an intrinsic difference between the ways in which chemical sensitizers and full protein antigens respectively stimulate the antibody-producing mechanism.

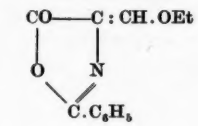
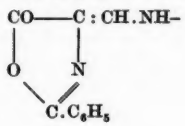
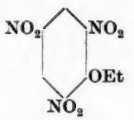
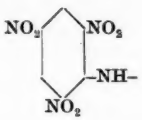
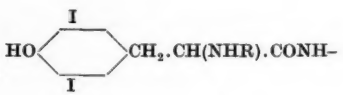
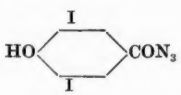
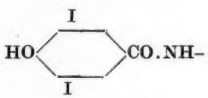
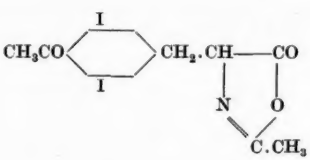
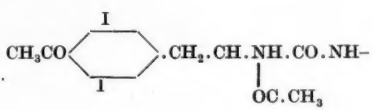
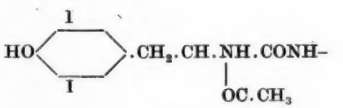
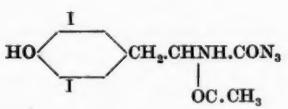
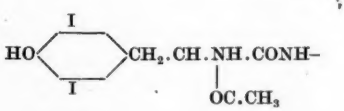
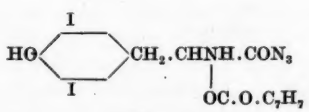
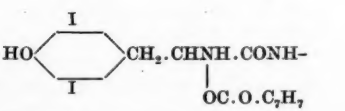
Many chemical compounds which act as sensitizers have one general feature in common : they are capable of reacting with a group or groups in the molecule of a protein to form a conjugate. A second type of sensitizer is not able itself to combine with protein, but may be metabolized in the body to a derivative possessing such powers of combination. There may be a third category of sensitizers which do not react chemically but form strong adsorption complexes with protein. In any case the conjugate or adsorption complex so formed will become antigenically distinct from the parent protein, and if the sensitizer gains access to the tissues of an animal the proteins of the latter may be rendered antigenic.

So far as the limited field of skin sensitization is concerned it may be regarded as proved that *in vivo* antigen formation of this type takes place. Typical powerful skin sensitizers are acid halides, some acid anhydrides, and benzene compounds containing a reactive halogen atom, all of which can combine with the amino groups of protein ; into this class also fall Tetryl and trinitrophenetole (*cf.* Gell, 1945). The arsenoxides in addition, which combine with protein SH groups, are sensitizing agents. The less direct type of sensitizer is exemplified by such compounds as *p*-phenylenediamine and polyhydric phenols (e.g. the active principle of poison ivy) ; these are probably oxidized in the body to quinones, which in turn react with proteins.

It is therefore easy to picture the mechanism by which sensitizing compounds form antigens within the body, and there seems to be no reason in principle why they should not, when applied in the appropriate manner, form antigens which will give rise to circulating antibodies of the precipitin type as distinct from the special antibodies which result from their intradermal injection. The object of the present work is to study the possibility of precipitin formation following the intraperitoneal injection of compounds selected for their known powers of combination with protein.

The substances selected are shown in Table I, which also indicates the type of linkage which will be formed by reaction with protein. Rabbits were given

TABLE I.

Substance injected.	Chemical formula.	Grouping formed in protein.
1. 2-Phenyl-4-ethoxy-methylene-oxazolone		
2. Trinitrophenetole		
3. Benzylchloroformate	$C_7H_7.O.CO.Cl$	$C_7H_7.O.CO.NH–$
4. Iodine		
5. 3 : 5-Diiodo-4-hydroxy-benzazide		
6. 2-Methyl-4(3′ : 5′-diiodo-4′-acetoxy-benzyl) oxazolone		
		and 
7. *N*-Acetyl-3 : 5-diiodo-tyrosine azide		
8. *N*-Carbobenzyloxy-3 : 5-diiodotyrosine azide		

courses of intraperitoneal injections of the different substances as indicated below (Table II) and the sera were subsequently tested, the test antigen in most cases being the product of reaction with gelatin of the substance employed. Although it is probable that the quantities of the substances used and the spacing of the injections were by no means optimal, it will be seen from the results recorded in the experimental portion of this paper that sera with easily measurable precipitin content were produced against seven out of the eight compounds employed; in some cases the sera may be considered quite comparable in strength with those which can be raised against full protein antigens. Hence the possibility that full precipitin formation may result from the introduction into the body of suitable simple chemical substances is definitely established.

EXPERIMENTAL.

Nature and Type of Reactivity of Simple Substances Used as Antigens.

I. 2-*Phenyl*-4-*ethoxymethylene-oxazolone.*

This substance, which was submitted to us for testing because of the strong sensitization which it had caused among laboratory workers, was one of a series synthesized during work on the chemistry of penicillin, and was kindly supplied by Dr. A. H. Cook, of the Imperial College of Science and Technology. It was found to be both a mild direct irritant on application to the epilated skins of guinea-pigs, at a concentration of M/10 or more, and an extremely powerful sensitizer.

The oxazolone ring of this compound is stabilized by the double bond at position 4; reaction with protein takes place by elimination of alcohol between the ethoxymethylene group and free $-NH_2$ groups. This condensation occurs readily at pH$>$8.

II. *Trinitrophenetole.*

This substance was found to be a strong sensitizer during work with the allied substance Tetryl (Gell, 1945). It is the ethyl ether of 2 : 4 : 6-trinitrophenol (picric acid), and reacts with proteins at pH 8–9 by elimination of alcohol forming a N-2 : 4 : 6-trinitrophenyl derivative.

III. *Benzyl chloroformate.*

This is a familiar stable acylating agent for amino-groups (Bergmann and Zervas, 1932). It is a fairly good skin sensitizer. Owing to its possible reactivity with alcoholic -OH it was not administered in propylene glycol solution as were the other antigens, but in acetone or paraffin.

IV. *Iodine.*

Iodine reacts with proteins chiefly by substitution of the tyrosine groups to give 3 : 5-diiodotyrosine, which is the only grouping taken into account in the present experiments; the test antigen used was prepared by coupling N-acetyl-3 : 5-diiodotyrosine azide with gelatin. After intradermal injections with iodine, animals are anaphylactically sensitized to diiodotyrosyl proteins or to iodinated proteins, but we have not found definite contact sensitivity to iodine itself.

Neither iodine nor any other of the iodine-containing antigens used in this work produced in the animals any obvious symptoms suggesting an effect on thyroid function.

V. 3 : 5-*Diiodo*-4-*hydroxybenzazide.*

This was found to be an excellent sensitizer. There is wide mutual cross-reaction between sera and antigens made from it and from N-acetyl-3 : 5-diiodotyrosine azide, as might be expected. The azide reacts with proteins like an acid halide, but is surprisingly stable to alkali (*cf.* preparation of Antigen No. 112).

Preparation. 3 : 5-*Diiodo*-4-*hydroxybenzhydrazide.*—Methyl 3 : 5-diiodo-4-hydroxybenzoate (4 g.) was heated under reflux for 2 hours with 50 per cent hydrazine hydrate (10 c.c.). The mixture was cooled, treated with sulphuric acid (17·8 c.c. of 5·05 N) and diluted with water. The crystalline precipitate was collected, washed with water and dried. The yield was 3·3 g. (82·5 per cent). (Found : N, 7·2 per cent. $C_7H_6O_2N_2I_2$ requires N, 6·9 per cent.)

3 : 5-*Diiodo*-4-*hydroxybenzazide.*—The above hydrazide (0·4 g.) was dissolved with gentle warming in a mixture of N hydrochloric acid (1·5 c.c.) and glacial acetic acid (7 c.c.) ; the solution was diluted with water (3 c.c.) and chilled in ice. Sodium nitrite (0·9 c.c. of 1·1 M) was then added to the stirred solution ; the azide started to crystallize when about one-third of the nitrite had been added. At the end of the addition the mixture was kept for 15 min. at 0° and the azide then collected, washed with water and dried. The yield was 0·35 g. (85 per cent) of cream-coloured needles, m.p. 122° (decomp.). (Found : N, 10·2 per cent. $C_7H_3O_2N_3I_2$ requires N, 10·1 per cent.)

VI. 2-*Methyl*-4(3′ : 5′-*diiodo*-4′-*acetoxybenzyl*)-*oxazolone.*

This substance is a fairly good skin sensitizer. Although it contains the same ring system as I, it reacts differently, the oxazolone ring behaving like an acid anhydride and forming an amide linkage with protein amino groups. Even under the mildly alkaline conditions of the coupling reaction *in vitro* a part at least of the O-acetyl will be removed. Rough experiments with the sera suggest that this may also occur in the body, antibodies being directed against the diiodophenolic grouping as well as against its acetyl derivative.

Preparation. ON-Diacetyl-l(+)3 : 5-diiodotyrosine.—*l*(+)3 : 5-Diiodotyrosine (8·66 g.) was dissolved in aqueous sodium hydroxide (70 c.c. of 2N) and treated with acetic anhydride (8 c.c.), which was added in portions with shaking and cooling. After 15 min. at room temperature the mixture was acidified with 5 N hydrochloric acid. An oil was precipitated which soon crystallized ; re-crystallization by solution in cold alcohol followed by dilution with water gave 8·85 g. (86 per cent) of colourless needles, m.p. 159°. (Found : I, 49·1 per cent ; $C_{13}H_{13}O_5NI_2$ requires I, 49·2 per cent.)

2-*Methyl*-4(3′ : 5′-*diiodo*-4′-*acetoxy-benzyl*) *oxazolone.*—ON-Diacetyl-diiodotyrosine (4·1 g.) was heated on the steam-bath for 20 min. with acetic anhydride (41 c.c.). The acetic anhydride was then evaporated under

diminished pressure and the residue dissolved in boiling benzene from which it separated in colourless prisms, m.p. 140°. The yield was 3·0 g. (76 per cent). (Found : I, 50·8 per cent. $C_{13}H_{11}O_4NI_2$ requires I, 50·9 per cent.)

VII. *N-Acetyl-3 : 5-diiodotyrosine azide.*

This will not elicit contact sensitivity but gives fairly good anaphylactic sensitivity ; some animals sensitized with this substance give a reaction on contact with compounds such as V and VI, suggesting that the lack of typical contact sensitivity is due to poor penetration or too great instability of the antigen. This compound reacts with protein with introduction of N-acetyl-3 : 5-diiodotyrosyl groups in peptide linkage.

Preparation. ON-Diacetyl-l(+)-3 : 5-diiodotyrosine ethyl ester.—*l*(+)-3 : 5-Diiodotyrosine ethyl ester hydrochloride (1 g.) was heated on a boiling water-bath for 50 min. with fused sodium acetate (0·25 g.) and acetic anhydride (2 c.c.). The solution was cooled somewhat and diluted with water. The oil which was precipitated soon crystallized ; the solid was collected and re-crystallized from 50 per cent acetic acid. The yield was 1·08 g. (98·5 per cent) of colourless needles m.p. 113–115°. (Found : I, 46·8 per cent. $C_{15}H_{17}O_5NI_2$ requires I, 46·6 per cent.)

N-Acetyldiiodotyrosine hydrazide.—The diacetyl ester (1·09 g.) was boiled under reflux for 1½ hours with alcohol (5 c.c.) and 50 per cent hydrazine hydrate (2 c.c.). The solution was evaporated to a syrup under diminished pressure ; the residue was taken up in hot water and the solution neutralized to litmus with hydrochloric acid. The product crystallized on cooling in a yield of 0·8 g. (75 per cent). It was recrystallized from water from which it separated in spear-shaped crystals m.p. 198° (decomp.). (Found : N, 8·8 per cent. $C_{11}H_{13}O_3N_3I_2$ requires N, 8·6 per cent.)

N-Acetyldiiodotyrosine azide.—The above hydrazide (0·53 g.) was dissolved in *N* hydrochloric acid (1·5 c.c.) and 50 per cent acetic acid (10 c.c.) ; sodium nitrite (0·9 c.c. of 1·1 M) was added slowly to the well stirred and chilled solution. The azide crystallized in colourless needles during the addition. The product was collected, washed with ice-cold 30 per cent acetic acid and dried. The yield was 0·48 g. (89 per cent). On slow heating in a melting-point apparatus the compound melted indefinitely ; it decomposed explosively when a capillary tube containing a little of it was plunged into boiling water. The behaviour on heating made accurate nitrogen determination impossible. (Found : N, 10·3 ; I, 50·5 per cent. $C_{11}H_{10}O_3N_4I_2$ requires N, 11·2 ; I, 50·8 per cent.)

VIII. *N-Carbobenzyloxy-l-3 : 5-diiodotyrosine azide.*

This reacts with protein similarly to VII. When used as an antigen it produces anaphylactic sensitivity not only to the diiodophenolic grouping but to the carbobenzyloxy group as well. Precipitins were also produced by it in rabbits, but in this particular case the sera, after Seitz filtration, were too weak to be easily titrated and serological results have therefore been omitted.

Preparation. N-Carbobenzyloxy-l-3 : 5-diiodotyrosine ethyl ester.—Diiodotyrosine ethyl ester hydrochloride (2·2 g.) was suspended in chloroform (30 c.c.). The suspension was cooled in ice and salt and shaken vigorously with N sodium hydroxide (1 equiv.). A solution of benzyl chloroformate (1·9 g. : 100 per cent excess) in chloroform (20 c.c.) was added in portions with shaking; simultaneously sodium bicarbonate (0·93 g.) was added in portions. When practically all the solid had passed into solution the chloroform layer was separated, washed successively with dilute hydrochloric acid, aqueous sodium bicarbonate and water and dried over sodium sulphate. It was evaporated to dryness under diminished pressure, and the residue was crystallized by dissolving in hot chloroform and precipitating with ligroin. The yield was 2·2 g. (84 per cent). After recrystallization from alcohol it formed needles, m.p. 124°. (Found: I, 42·3 per cent. $C_{19}H_{19}O_5NI_2$ requires I, 42·7 per cent.)

N-Carbobenzyloxydiiodotyrosine hydrazide.—The above ester (1·2 g.) was boiled under reflux for 1½ hours with alcohol (10 c.c.) and 50 per cent hydrazine hydrate (2 c.c.). The solution was treated while hot with hydrochloric acid until just acid to Congo paper and diluted slightly with water. After chilling the precipitate was collected, washed and dried. The yield was 0·78 g. (67 per cent). Recrystallized from *n*-butyl alcohol it formed needles, m.p. 202–3° (decomp.). (Found: N, 7·4 per cent. $C_{17}H_{17}O_4N_3I_2$ requires N, 7·2 per cent.)

N-Carbobenzyloxydiiodotyrosine azide.—The hydrazide (0·58 g.) was dissolved in a mixture of N hydrochloric acid (1·5 c.c.) and glacial acetic acid (7 c.c.); the solution was chilled and stirred while sodium nitrite (0·9 c.c. of 1·1 M) was slowly added. The azide crystallized in needles and was collected, washed and dried. The yield was 0·4 g. (68 per cent). The compound decomposed explosively on sudden heating to 100°. Analysis showed that it was not quite pure. (Found: N, 8·7; I, 41·9 per cent. $C_{17}H_{14}O_4N_3I_2$ requires N, 9·5; I, 42·9 per cent.)

Preparation of Immune Sera.

The course of injections used with each of the various antigens is detailed in Table II. As will be seen, the usual course was quite short; in one case, which gave one of the best sera, the course was only 14 days, and indeed the first-course sera corresponding with several others were almost as good as the second-course. Young adult sandy-lop rabbits were used throughout. All the rabbits produced precipitins, but only the stronger serum of each pair was titrated, unless the sera were equally good, in which case they were combined. There was more variation between the strengths of the sera produced in response to the weaker antigens than there was between those produced against the stronger. A characteristic of precipitins against antigens of this type seems to be their transience; their titres were highest on the 4th or sometimes even on the 3rd day after the last injection, and had fallen markedly by the end of a week. The sera were kept for at least 2 weeks at 2° in the ice chest, with 0·1 per cent sodium azide as a preservative, and were Seitz-filtered a few days before testing; this seemed to have a slightly deleterious effect on all except No. 1, as judged roughly by a serum-dilution ring test. Heating had a similarly damaging effect, so none

except No. 1 was heated to inactivate complement. The amount of complement present in the precipitates must therefore be taken into account, since just detectable amounts were present in all sera in spite of ageing and filtration, but the amounts by weight must have been negligible.

Since the purpose of this work is to show only that tolerably strong sera may be provoked by intraperitoneal injection of antigens of the type used, the method of washing and preparing the precipitates is admittedly not as rigorous as would be required for properly quantitative data. In particular some dissociation and re-solution of the antigen-antibody complexes may have taken place, especially since temperature appears to have a considerable effect on the solubility of precipitates containing gelatin antigens. The precipitation at 37°C. was visibly weaker than that at room temperature, although we have made no quantitative studies on this point. Therefore the familiar commercial anti-pneumococcal horse serum, solution (No. 8), as issued for diagnostic work, has been estimated along with the others, in order to serve as a standard of comparison.

TABLE II.—*Course of Immunization of Rabbits.*

Rabbit No.	Course No.	Antigen Type (cf. Table I).	Antigen concentration.	Route.	No. injections.	Dose (c.c.) varying between.—	Total dose (c.c.).	Duration of injections.	Serum taken (days from last injection).	Rest between courses (days).	Serum No.
9	i	I*	*M*/10	I–P	9	0·2 –0·6	3·6	21	..	90	..
	ii		„	„	6	0·1 –0·5	2·1	14	3, 4, 5	..	1
16	i	II†	*M*/25	„	9	0·2 –0·6	3·6	21	4	10	2
	ii		*M*/10	„	6	0·25–0·5	2·75	14	4	..	
23	i	III‡	*M*/100	„	9	0·2 –0·6	3·6	21	..	10	..
	ii		*M*/10	„	7	0·15–0·3	1·65	14	..	7	..
	iii		„	I–D	6	0·1 –0·3	1·15	14	4	..	3
28	i	IV§	*M*/25	I–P	6	0·2 –0·4	1·80	14	..	16	..
	ii		*M*/10	„	7	0·25–0·5	2·75	14	..	16	..
	iii		„	„	6	0·1 –0·2	1·00	14	3	..	4
34	i	V	„	„	7	0·3 –0·6	3·30	14	4	..	5
35	i		„	„	6	0·3 –0·6	3·00	14	3	..	
21, 26	i	VI	„	„	9	0·2 –0·6	3·60	21	..	10	..
	ii		„	„	6	0·4 –0·8	4·40	14	4	..	6
14, 31	i	VII	„	„	9	0·2 –0·6	3·6	21	..	21	..
	ii		„	„	6	0·4 –0·8	3·6	14	2, 3	..	7

Control: Anti-pneumococcus (Type III) Horse serum (Parke-Davis: Batch No. 066763B Agglutination titre 1/60). 8

* Both rabbits produced strong first- and second-course sera to this antigen.

† Sera of similar potency from both first and second courses were combined.

‡ The first course using the antigen at *M*/100 in paraffin produced no precipitins. The substance was therefore dissolved in acetone at *M*/10 for I–P injections, which in the small volumes used were quite well tolerated; this second course produced a weakly positive serum. The third course consisted of small doses in acetone intradermally, and a moderately good serum was produced.

§ The three courses with this antigen produced sera of increasing potency.

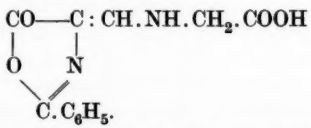
Preparation and Analysis of Test Antigens.

In all preparations from gelatin, Coignet's Gold Leaf gelatin was used; the alkali employed was freshly made up from pellets of sodium hydroxide to avoid traces of silicate which may give non-specific precipitation with serum. A mol. wt. of 18,000 was assumed for the gelatin.

1. *Antigen* 89. *Reaction product of 2-phenyl-4-ethoxymethylene-oxazolone with gelatin.*

Gelatin (1 g.) was dissolved in hot water (50 c.c.), the cooled solution was made alkaline to phenolphthalein by addition of N sodium hydroxide and mixed with alcohol (50 c.c.), more alkali being added to restore the red colour of the indicator. A solution of 2-phenyl-4-ethoxymethylene oxazolone (0·5 g.) in alcohol (15 c.c.) was added slowly to the stirred solution of gelatin at room temperature, N sodium hydroxide also being added as required to maintain alkalinity to phenolphthalein. The mixture was kept for 30 minutes and then acidified with acetic acid and mixed with several volumes of alcohol. The precipitate was collected at the centrifuge, extracted several times with alcohol and dried *in vacuo* over phosphorus pentoxide; it had N 14·5 per cent.

Analysis.—It was found that the oxazolone group gave a blue colour with the phenol reagent of Folin and Ciocalteu (1927) and use was made of this fact as follows: The oxazolone was coupled with glycine dissolved in 1 equiv. of aqueous sodium hydroxide to give the compound



This, after crystallization by solution in cold alcohol and dilution with water, had m.p. 192° (decomp.). (Found: N, 11·3 per cent. $C_{12}H_{10}O_4N_2$ requires N, 11·4 per cent.) A calibration curve for the colour given by this compound with Folin's reagent, measured in the Pulfrich step-photometer using filter S53, was prepared.

For the determination 0·5 g. portions of antigen 89 and of gelatin were separately dissolved in water and precipitated with two volumes of alcohol; after 24 hours at 0° the supernatant liquids were decanted and the residual gels shaken with acetone. The resulting powders were dried in the same vacuum desiccator over phosphorus pentoxide.

The extinction coefficients with filter S53 of the colours produced by treating 50 mg. portions of the antigen and of gelatin with Folin's reagent were then measured; from the values obtained the amount of oxazolone could be read off from the standard curve prepared as described above. The oxazolone content of antigen 89 was thus found to be 5·52 per cent.

Desiccation over phosphorus pentoxide does not completely remove water from gelatin. For subsequent calculations it was necessary to ascertain the true nitrogen and hapten contents of the anhydrous substance; these were calculated as follows:

The nitrogen content of gelatin was taken to be 17·5 per cent; in this present case the nitrogen content of the hapten residue was 8·14 per cent. If *a* be the

g. gelatin in 100 g. of the antigen as analysed and which contains 14·5 per cent total N and 5·52 per cent hapten residue, we then have—

$$a \times 17{\cdot}5 + 5{\cdot}52 \times 8{\cdot}14 = 100 \times 14{\cdot}5$$
whence $a = 80$.

100 g. of the antigen as analysed therefore contain 80 g. of gelatin and 5·52 g. hapten residues, whence the true nitrogen and hapten contents of the anhydrous material are $14{\cdot}5 \times 100/85{\cdot}52$ or 16·95 per cent and $5{\cdot}52 \times 100/85{\cdot}52$ or 6·46 per cent respectively.

The figures given for the nitrogen and hapten contents of the other gelatin antigens described below are the corrected values obtained by analogous calculations which are not repeated.

2. *Antigen* 41. *Picrylgelatin.*

Gelatin (2 g.) was dissolved in warm water (30 c.c.). The temperature was raised to 60–70° and picryl chloride (1 g.) in alcohol (20 c.c.) was added drop-wise from a pipette, the solution being stirred and kept alkaline to cresol red by periodic additions of *N* sodium hydroxide. The solution was dialysed against running water overnight and acidified with acetic acid. The precipitate was redissolved in 0·9 per cent sodium chloride, reprecipitated with acetone and dried at 37°. It was redissolved, adjusted to pH 7·5, precipitated with 1·5 to 2 volumes of alcohol and kept in the ice chest overnight; this process was repeated and the product was finally collected, washed with acetone and dried in a vacuum desiccator over sulphuric acid. It formed a fine dark orange-coloured powder, having N (corrected) 17·7 per cent.

Analysis.—The picryl groups in this antigen were estimated by a modification of the method used by Gell (1945). An appropriate amount of the picrylgelatin was hydrolysed and the hydrolysate reduced, diazotized and coupled with α-naphthylethylenediamine as there described. The extinction coefficient of the coloured solution was measured in the Pulfrich step-photometer using filter S53, and the amount of the picryl groups was read off from a standard curve which had been prepared from picrylglycine. The picrylgelatin was found to contain 6·4 per cent picryl residue.

3. *Antigen* 86. *Carbobenzyloxygelatin.*

Gelatin (3 g.) was dissolved in hot water (150 c.c.); after cooling the solution was mixed with dioxan (150 c.c.) and enough *N* sodium hydroxide to maintain alkalinity to phenolphthalein. The stirred mixture was gradually treated with benzylchloroformate (0·7 g.) in dioxan (7 c.c.) alkalinity being maintained by parallel addition of N sodium hydroxide. The product was isolated by acidification with acetic acid; it was collected, extracted several times by grinding with alcohol and dried. It had N (corrected) 15·1 per cent.

Analysis.—A weighed amount of the carbobenzyloxygelatin was boiled under reflux with 7 N sulphuric acid for 1·5 hours, the carbon dioxide evolved being swept with a stream of nitrogen into a train of three tubes containing in all 50 c.c. N/70 barium hydroxide; the baryta solutions were combined, filtered from barium carbonate and titrated with N/70 hydrochloric acid. Previous experiments had shown that carbobenzyloxyglycine gave a quantitative yield of

carbon dioxide by this method, whilst negligible amounts of carbon dioxide were evolved from gelatin itself. Duplicate determinations gave for the carbobenzyloxygelatin 12 per cent (corrected) of carbobenzyloxy residues.

4. *Antigen* 112. 3 : 5-*Diiodo*-4-*hydroxybenzoylgelatin.*

Gelatin (1 g.) was dissolved in warm water (15 c.c.) with enough *N* sodium hydroxide to make alkaline to phenolphthalein ; 0·5 c.c. more of the sodium hydroxide was then added, followed by 3 : 5-diiodo-4-hydroxybenzazide (0·1 g.) in acetone (4 c.c.). The solution was left overnight and by next morning had set to a jelly : this was melted by gentle warming and acidified with acetic acid ; on addition of a large excess of acetone the precipitate hardened. It was collected, repeatedly extracted with acetone, redissolved in water with the aid of alkali, and again re-precipitated by acidification and addition of acetone. The corrected analytical figures were N 16·3 per cent and diiodohydroxybenzyl residue 6·94 per cent.

5. *Antigen* 113. *ON-Diacetyl*-3 : 5-*diiodotyrosylgelatin.*

This was prepared from gelatin (1 g.) and 2-methyl-4-(3′ : 5′-diiodo-4′-acetoxybenzyl) oxazolone (0·5 g.) by the same technique as employed for antigen 112 (see above). The product had N (corrected) 16·7 per cent and ON-diacetyldiiodotyrosyl residues 5·35 per cent.

6. *Antigen* 110. *N-Acetyl*-3 : 5-*diiodotyrosylgelatin.*

This was again prepared by the same technique from gelatin (1 g.) and N-acetyl-3 : 5-diiodotyrosylazide. The product had N (corrected) 15·8 per cent and N-acetyldiiodotyrosyl residue 11·8 per cent.

SSS. III.—This was prepared from a virulent type III Pneumococcus culture (N.C.T.C. 6366) passaged through mice. The culture fluid was concentrated by freezing to 20 per cent of its original volume, and precipitated with alcohol in the usual way. The crude precipitates from a number of preparations were then dissolved to give a 0·5 per cent solution in water containing about 5 per cent potassium acetate and 5 per cent sodium benzoate. The solution was acidified with 5 N hydrochloric acid added very slowly in the cold under mechanical stirring to pH about 2·7 (Congo paper purplish red), when the thick precipitate of benzoic acid suddenly became " curdy " and could easily be filtered off. The filtrate was precipitated with alcohol and the whole process repeated. The final precipitate re-precipitated from water with alcohol was hardened in absolute alcohol and dried over PCl_5. N content (Kjeldahl) 0·13 per cent (not corrected). This is a satisfactory and simple method of preparing samples of SSS. III of fairly high purity but the exact conditions, yield, etc., have not yet been worked out. The low pH does not appear to affect SSS. III, though other pneumococcal carbohydrates might be damaged.

For the precipitin tests, solutions of the test antigens were made up in saline at an approximate concentration of 50 mg./c.c. except in the case of antigen 89, where less was inadvertently taken. The true concentrations were calculated from determinations of the total nitrogen content of the solutions. Various other data relevant to these antigens were also calculated from their known nitrogen and hapten contents and are given in Table III.

TABLE III.—*Analytical Data on Gelatin Test Antigens.*

Antigen No.	41	86	89	110	112	113
Nature of reacting group	Trinitrophentole	Benzylchloroformate	2-Phenyl-4-ethoxymethylene oxazolone	N-Acetyldiiodotyrosine azide	3 : 5-Diiodo-4-hydroxybenzazide	2-Methyl-4-(3 : 5-diiodo-4-acetoxybenzyl)-oxazolone
Mol.wt.of hapten residue	212	135	172	458	373	500
μg. N/c.c. of solution	764	704	428	670	734	742
μg. Antigen/c.c. solution	4320	4670	2525	4240	4500	4440
Hapten content of antigen per cent	6·4	12·0	6·46	11·8	7·0	5·35
μg. Gelatin/c.c. solution	4040	4100	2360	3780	4180	4210
μmol. antigen/c.c. solution based on gelatin content	0·225	0·228	0·131	0·210	0·234	0·234
No. of hapten groups per mol. gelatin to nearest integer	5	16	7	5	3	2

Precipitin Tests, Methods, Estimation and Results.

The details of the precipitin tests are summarized in Table IV. The antigen dilutions were placed in 2 in. by $\frac{5}{16}$ in. test-tubes calibrated at 2·5 c.c. and saline added to give a final volume (after addition of serum) of 2·5 c.c. (except in the case of serum 24, where the volume was 3 c.c.). The requisite amount of serum was added from a pipette, and the solutions rapidly mixed by inverting the tube four times on to a small slip of cellophane. The tubes were then left at room temperature, with frequent brisk shaking by hand, until precipitation was well advanced in all tubes, or for a minimum of two hours. With most of the sera precipitation occurred in the majority of the tubes within ten minutes. The tubes were capped with silver paper and placed in the ice-chest for five days. They were then centrifuged in an angle centrifuge, drained thoroughly, washed four times at the centrifuge with 5 c.c. of cold saline and left inverted on blotting-paper in the ice-chest overnight. Before the third washing an attempt was made to break up the precipitate with a glass rod, but this was rendered very difficult by the great toughness and stickiness of the deposit, apparently owing to the use of gelatin as an antigen. The combined supernatants from the last wash were tested with Folin's reagent and were always found to be free from protein.

The deposits, after draining overnight, were dissolved in 0·3 c.c. $N/_{10}$ sodium hydroxide and diluted up to the 2·5 c.c. mark with water. Aliquots of 2 c.c. were taken from each sample and transferred to a 50 c.c. flask. 6 c.c. of N sodium hydroxide were then added and 3 c.c. of Folin's phenol reagent freshly diluted to one-third concentration. After 5 to 10 minutes the extinction coefficients of the solution were determined in a Pulfrich step-photometer using filter S53 and 0·5, 1 or 2 cm. cells, according to the intensity of the colour.

To determine the amount of antibody protein a standard curve was made, using a rabbit γ-globulin solution (95 per cent pure by electrophoresis)* containing 6·2 per cent protein (by Kjeldahl estimation). This was diluted to 1/50 and

* This determination was kindly carried out for us by Dr. D. Bunn.

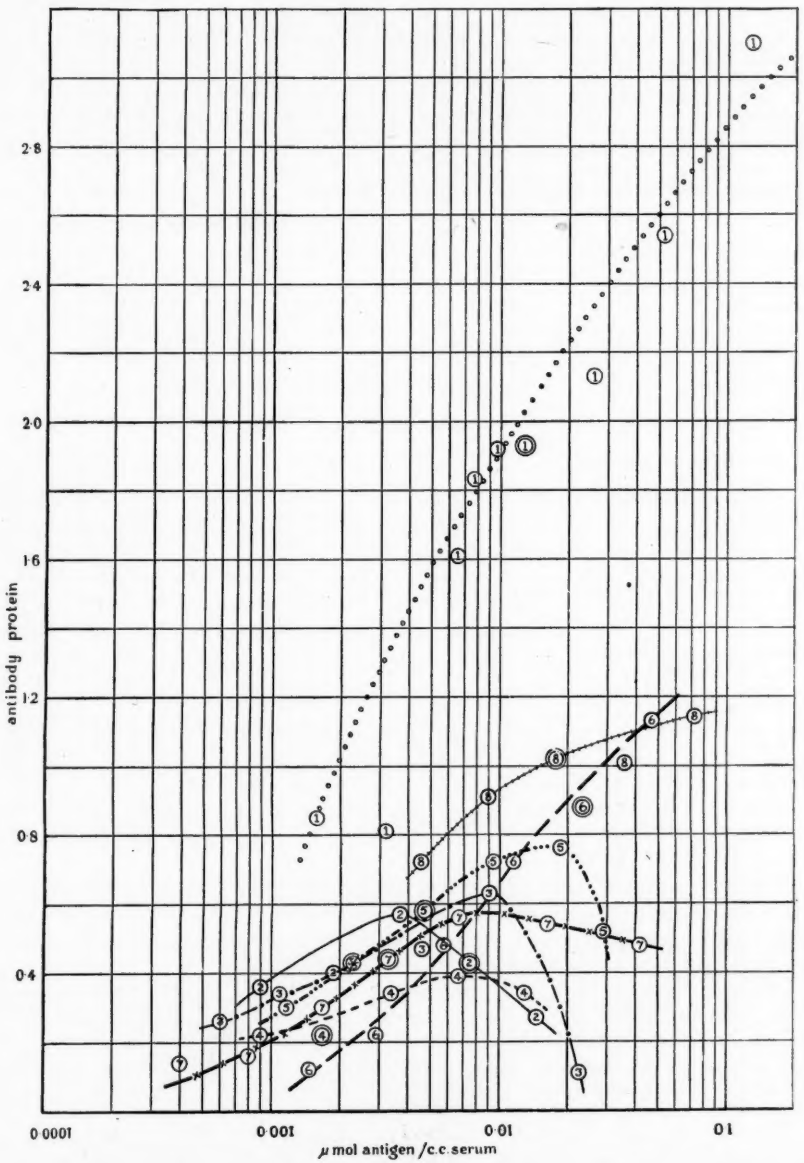


FIG. 1.—Relationship of antibody protein in mg. per c.c. of serum to log of μmol conjugate. (position of curve 8 is arbitrary).

TABLE IV.—*Estimation of*

		Antigen : SSS. III. Serum : No. 8 *(anti-*				
i	Antigen solution, c.c., in all tubes	0·5	..	..	..	..
ii	Serum, c.c., in all tubes	1	..	..	..	..
iii	Antigen, μmol./c.c., undiluted	0·225 (arbitrary figure)			..	..
iv	Antigen dilution	1·56	3·125	6·25	12·5	25
v	Antigen, μmol./c.c. serum	..	..	..	..	..
vi	$E_{1\text{ cm.}}$ for antibody/c.c. serum, corrected (see text)	0·322	0·284	0·289	0·255	0·210
vii	Antibody protein, mg./c.c. serum	1·14	1·01	1·02	0·91	0·72
viii	Antibody excess (A) or antigen excess (G)	G	G	G opt.	A	A
		Antigen : No. 41 ; *sym-Trinitrophenyl- Serum :*				
i	Antigen solution, c.c., in all tubes	0·5	..	..	..	..
ii	Serum, c.c., in all tubes	1	..	..	..	..
iii	Antigen, μmol./c.c., undiluted	0·225	..	..	..	..
iv	Antigen dilution	7·5	15	30	60	120
v	Antigen, μmol./c.c. serum	0·0150	0·0075	0·0037	0·0019	0·0009
vi	$E_{1\text{ cm.}}$ for antibody/c.c. serum, corrected (see text)	0·094	0·134	0·172	0·127	0·117
vii	Antibody protein, mg./c.c. serum	0·27	0·43	0·57	0·40	0·36
viii	Antibody excess (A) or antigen excess (G)	G	G opt.	..	..	A
		Antigen : No. 110 ; N-*Acetyl-diiodotyrosyl-* (*anti-iodine*).				
i	Antigen solution, c.c., in all tubes	0·4	..	..	..	..
ii	Serum, c.c., in all tubes	2	..	..	..	..
iii	Antigen, μmol./c.c., undiluted	0·210	..	..	..	..
iv	Antigen dilution	3·125	6·25	12·5	25	50
v	Antigen, μmol./c.c. serum	0·0134	0·0067	0·0034	0·0017	0·0009
vi	$E_{1\text{ cm.}}$ for antibody/c.c. serum, corrected (see text)	0·111	0·126	0·111	0·079	0·079
vii	Antibody protein, mg./c.c. serum	0·34	0·39	0·34	0·22	0·22
viii	Antibody excess (A) or antigen excess (G)	G	G	G	G opt.	..
		Antigen : No. 113 ; ON-*Diacetyl-diiodotyrosyl-*				
i	Antigen solution, c.c., in all tubes	0·5	..	..	..	..
ii	Serum, c.c., in all tubes	2	..	..	..	..
iii	Antigen, μmol./c.c., undiluted	0·234	..	..	..	..
iv	Antigen dilution	1·25	2·5	5	10	20
v	Antigen, μmol./c.c. serum	0·0468	0·0234	0·0117	0·0058	0·0029
vi	$E_{1\text{ cm.}}$ for antibody/c.c. serum, corrected (see text)	0·318	0·251	0·211	0·149	0·080
vii	Antibody protein, mg./c.c. serum	1·13	0·88	0·72	0·48	0·22
viii	Antibody excess (A) or antigen excess (G)	G	G opt.	A	A	A

Note.—The italicized figures are the values for the extinction coefficient determined in these figures the proportionate corrections

1/100, and amounts of these dilutions ranging from 0·1 to 2 c.c. were taken, made up to 2 c.c. where necessary with water, and estimated by the technique described above. By means of this curve the weight of antibody protein was read directly from the extinction coefficients corrected as in Table IV.

The supernatants from the precipitates were tested by the ring technique against the undiluted serum for antigen excess, and against 1/25 and 1/125 dilutions of the antigen solution for serum excess, readings being taken up to one hour, at room temperature. With sera Nos. 3, 5, 6, 7 and 8, either antigen or antibody was detected in every supernatant, but not both ; there was therefore a good " equivalence zone " covering a twofold dilution of antigen. With sera Nos. 2 and 4, there were tubes where neither antigen nor antibody were detectable, probably owing to the difficulty of detecting small amounts of antibody from these rather weak sera. Serum No. 1 was the only one in which more closely spaced dilutions were tested in the optimal area, and here there was a definite

ntibody Content of Sera.

neumococcal).		*Antigen : No.* 89 ; 2-*Phenyloxazolone–*				*Serum : No.* 1.				
..	..	1	..	..	..	..	..	..	..	..	..
..	..	1	..	..	..	..	..	..	..	..	..
..	..	0·131	..	..	..	..	..	..	..	..	..
..	*1·56*	1	2·5	5	10	13·5	17	20	40	80	*10*
..	..	0·1310	0·0524	0·0262	0·0131	0·0097	0·0077	0·0065	0·0032	0·0016	..
..	*0·003*	0·830	0·678	0·577	0·525	0·523	0·499	0·442	0·235	0·244	*0·045*
..	..	3·10	2·54	2·13	1·93	1·92	1·83	1·61	0·81	0·85	..
..	..	G	G	G	G opt.	GA	GA	A	A	A	..
No. 2.		*Antigen : No.* 86 ; *Carbobenzyloxy–*				*Serum : No.* 3.				
..	..	0·5	..	..	..	..	..	..	..	..	..
..	..	2	..	..	..	..	..	..	..	..	..
..	..	0·228	..	..	..	..	..	..	..	..	..
..	*1·25*	2·5	6·25	12·5	25	50	100	..	..	..	*6·25*
..	..	0·0228	0·0091	0·0046	0·0023	0·0011	0·0006	..	..	..	..
..	*0·022*	0·065	0·187	0·146	0·134	0·111	0·091	..	..	..	*0·026*
..	..	0·15	0·63	0·47	0·43	0·34	0·26	..	..	..	..
..	..	G	G	G	G opt.	A	A	..	..	..	..
Serum : No. 4		*Antigen : No.* 112 ; 3 : 5-*Diiodo-4-hydroxylbenzoyl–*				*Serum : No.* 5.				
..	..	0·5	..	..	..	..	..	..	..	..	..
..	..	2	..	..	..	..	..	..	..	..	..
..	..	0·234	..	..	..	..	..	..	..	..	..
..	*6·25*	2	3·125	6·25	12·5	25	50	..	..	..	*3·125*
..	..	0·0293	0·0187	0·0094	0·0047	0·0023	0·0012	..	..	..	..
..	*0·048*	0·159	0·220	0·211	0·174	0·135	0·102	..	..	..	*0·036*
..	..	0·52	0·76	0·72	0·58	0·43	0·30	..	..	..	..
..	..	G	G	G	G	G opt.	A	..	..	..	*0·048*
Serum : No. 6.		*Antigen : No.* 110 ; N-*Acetyldiiodotyrosyl–*				*Serum : No.* 7.				
..	..	0·4	..	..	..	..	..	..	..	..	..
..	..	2	..	..	..	..	..	..	..	..	..
..	..	0·210	..	..	..	..	..	..	..	..	..
40	*2·5*	1	2·5	6·25	12·5	25	50	100	..	..	*6·25*
0·0015	..	0·0420	0·0168	0·0067	0·0033	0·0017	0·0008	0·0004	..	..	..
0·053	*0·045*	0·144	0·166	0·171	0·136	0·102	0·067	0·060	..	..	..
0·12	..	0·48	0·54	0·56	0·44	0·30	0·16	0·14	..	..	..
A	..	G	G	G	G opt.	A	A	A	..	..	..

tubes in which serum was replaced by saline at the stated dilution of antigen. From have been applied as explained in the text.

overlap in two tubes. This would possibly have been found with other sera by a similar technique.

The results of the analysis are detailed in Table IV and shown graphically in Fig. 1. In Table IV the first three lines describe the experimental set-up. Line iv shows the dilution of the test antigen solution used and line v the fraction of a μmol. of antigen, per c.c. of serum, in this tube ; for instance, if 0·5 c.c. of antigen and 2 c.c. of serum were used, a 1/1 dilution will be the value given in line iii divided by four, and similarly for the other dilutions. The antigens themselves give small amounts of colour with Folin's reagents. In each case the extinction coefficient of this colour has been determined at a certain dilution of antigen in the absence of serum, and these values appear in italics in Table IV. From the figures thus obtained the antigen corrections at the optima were deduced ; proportional corrections for tubes in the regions of antibody and antigen excess respectively were calculated as follows : In the region of antibody excess,

up to and including the first tube showing slight antigen excess marked (G opt in line viii), the theoretical reading for the total amount of antigen present in the system was deducted, on the assumption that it was virtually all present in the precipitate. For precipitates in the region of antigen excess the ratio of antibody to antigen in the G opt. tube was assumed to hold throughout and a correction proportional to this was applied. This seemed to be a fair maximal deduction, since " incomplete " antibody carried down only in antigen excess was likely to react with less rather than more antigen, proportionally, than that precipitating at the optimum. Line vi shows the extinction coefficients per c.c. serum corrected by subtracting the appropriate antigen correction from the observed $E_{1\text{ cm.}}$; in line vii are shown the values for antibody protein per c.c. derived from the corrected figures for $E_{1\text{ cm.}}$ The last line (viii) shows antigen (G) or antibody (A) excess. A " serum control " was included in each system; but the results were so variable, and usually so small, that this control was taken to indicate only the margin of error of the method, and no correction for it has been included.

Fig. 1 shows the antibody protein values (line vii, Table IV) plotted against the log of the values for μmol. conjugate present (line v, Table IV). An arbitrary position has been allotted to curve VIII (for the control serum against the penumococcal polysaccharide) for purposes of comparison.

DISCUSSION.

It is clear that the work described here has a bearing on two current problems of immunology. The first problem, which is in fact now largely terminological, is that of the protein nature of antigens; the second is the question of " auto-antibodies," i.e. antibodies directed against an animal's own protein made antigenic.

We have frequently referred to the simple chemical substances studied in this work, such as acid azides, as " antigens "—a practice which might be taken to be a rejection of the view that antigens must necessarily be proteins. From the terminological point of view this is so, but the difficulty disappears if we designate the simple compounds as " pro-antigens "; from the point of view of the process involved, our experiments may be regarded as carrying out *in vivo* a manipulation which is normally done *in vitro*, viz. the combination of a chemically reactive hapten with a protein to form a fully antigenic conjugate. This entails no new principle, the actual stimulation of antibody-production being still, in all probability, the result of conjugation of the proantigenic determinant groups with the proteins of tissue fluid to form fully antigenic complexes. There is another possibility, on which we have no direct evidence either way, namely that the chemical substances (perhaps, since they are mostly insoluble in aqueous media, in microcrystalline condition) are taken up by phagocytes before any reaction has occurred, and that conjugation takes place inside the cell, either with defined intracellular proteins or with groups on the protein complex of the cytoplasm as a whole. It is possible moreover that these are the actual cells responsible for the formation of the antibodies.

Intracellular conjugation of the second type can be readily envisaged for some of the substances used, owing to their relative slowness of reaction either with water or with protein. On the other hand, with iodine at least (and perhaps with some of the less stable of the other substances), reaction with *any* available

protein will be almost immediate, and the antigen in these cases must be assumed to be at least mainly chemically altered peritoneal-fluid-proteins.

Although, therefore, the present work contributes, so far as we are aware, the first clear-cut evidence that precipitins can be formed in response to injection of simple chemical compounds, there is nothing particularly surprising in the results which we have obtained. They fall into line with, and are indeed what would be expected from, the view that a full antigen must contain protein. The real difficulty in the way of accepting the general validity of this view is not offered by our observations with simple but highly reactive compounds of carefully selected types; a more serious difficulty is to explain the antigenicity of high-molecular polysaccharides of bacterial origin which now seems to be established, at least for the specific soluble substances of pneumococci. Rigid adherence to the doctrine that a full antigen must contain protein would imply that these substances must undergo some form of combination with body proteins. Chemical combination in the ordinary sense can hardly be envisaged, but a specific adsorption remains a possibility. A hint that this may be the true explanation is afforded by the success of Morgan (1943) in making highly specific antigens by mixing high-molecular polysaccharides with the protein component of the Shiga antigen in formamide solution; here it does not seem likely that chemical reaction occurs between the polysaccharide and the protein.

Whatever may prove ultimately to be the true explanation of the antigenicity of certain polysaccharides, our own results seem to make it perfectly clear that homologous proteins may be rendered antigenic *in vivo*. It is already known that certain specialized proteins, such as lens protein, are antigenic in the homologous species, and it is also known that other proteins, not normally antigenic in the species from which they are derived, can be made so *in vitro*. Thus " picrylated " homologous red cell stromata were found by Landsteiner and Chase (1941) to be antigenic in the homologous species. In this laboratory Gell (1945) showed that satisfactory anti-" picryl " sera could be produced in rabbits and guinea-pigs by means of antigens made from their own serum proteins. (It is interesting to note however that such antisera were definitely weaker than the serum described in this paper which resulted from injection of trinitrophenetole.) We have also raised a strong antiserum in rabbits by injection of the reaction product of 2-phenyl-4-ethoxymethylene oxazolone and rabbit serum protein.

Such *in vitro* experiments are subject to the general criticism that the proteins have been altered in two ways, by conjugation with a new chemical compound and by denaturation which inevitably accompanies chemical manipulation. In the *in vivo* experiments described in this paper such denaturation is certainly minimal, and unless it be argued that antibody-production in these experiments is not elicited by a conjugate with protein at all, it must be admitted that homologous protein can readily be made antigenic, since here there was no other protein available.

In spite of the fact that only two rabbits were used for each substance studied, it is tempting to try to correlate the relative effectiveness of the various " antigens " in producing precipitins with their chemical properties. With the exception of iodine all the compounds used react with protein by combination with NH_2 groups; they are all also susceptible to hydrolysis, particularly at high temperatures and high pH. By the proper selection of conditions *in vitro* it is possible to make these compounds react almost quantitatively with pure amino

acids even in aqueous solution, the optimum conditions being a low temperature and the minimum pH at which the amino group is undissociated. *In vivo* such ideal conditions will not obtain; the relatively high temperature will favour hydrolysis, and the relatively low pH will mean that fewer amino groups are undissociated and therefore available for combination. It is therefore reasonable to anticipate that those compounds which are hydrolysed with extreme ease will be relatively ineffective in forming antigens *in vivo*; the more stable ones will not only be more likely to react with amino groups before hydrolysis occurs, but they may also in part survive unchanged, as already suggested, until they are absorbed by phagocytes, in which they may find an environment more suitable for conjugation.

If we examine the figures for the maximal amounts of precipitated antibody (Table IV) which may reasonably be taken as indications of the relative efficiencies of the corresponding antigens, we find that 2-phenyl-4-ethoxymethylene oxazolone, 2-benzyl-4 (3 : 5-diiodo-4-acetoxyphenyl) oxazolone and 3 : 5-diiodo-4-hydroxybenzazide in that order produce the sera of highest titre. Trinitrophenetole should probably be included in this group of highly active antigens; it would have been so included had its gelatin derivative not failed (possibly owing to the solubility of the N-trinitrophenyl group) to behave like all the other test antigens in precipitating very much more antibody in the region of great antigen excess. The four compounds just mentioned are definitely more resistant to hydrolysis than benzylchloroformate and N-acetyldiiodotyrosine azide, which proved themselves to be less effective precipitinogens. The weakness of iodine in producing precipitins may be accounted for by the fact that according to Haurowitz and Appel (1939) far more iodine per mol. of protein is needed to convert a protein into a good antigen (for specific diiodophenolic groups) than is needed for other bulkier substituents with a determinant function which are less likely to be able to penetrate to the interior of a three-dimensional protein molecule. The general trend of our actual observations is therefore in accordance with the views expressed in the preceding paragraph.

Examination of the curves in Fig. 1 shows that most of the sera contain a proportion of antibody precipitable only in the region of antigen excess. Serum No. 2 (anti-picryl) forms an exception to this statement, and a possible reason for the behaviour of this particular serum has already been mentioned. The effect is present to a slight extent only in the weak serum No. 4 (anti-iodine) and in the anti-pneumococcal serum (No. 8), but is conspicuous in the strongly reacting sera Nos. 5, 6, and especially in No. 1, where it is extremely striking.

It thus appears that the presence of unusually large amounts of antibody precipitable only in the region of antibody excess is characteristic of the stronger antisera raised against pro-antigens of the type which we have used. Such "incomplete" antibody, it has been suggested, may be univalent, and hence unable to take part in lattice formation (Marrack, 1934). It is interesting to consider whether the presence of large amounts of this type of antibody may also be related to the chemical reactivity of the pro-antigens themselves.

We have given reasons above for supposing that the effectiveness of a pro-antigen is associated with a certain degree of resistance to hydrolysis; it is these relatively stable pro-antigens which produced the strongly reacting sera, containing much incomplete antibody which we are discussing. Such stability will not only, as already pointed out, enable the pro-antigen to react more com-

pletely with protein; it will also cause it to become more highly diluted by the peritoneal fluid, before reaction takes place. This will in turn mean the formation of a relatively large proportion of conjugated protein containing only one hapten group per molecule.

It is impossible to say whether such "univalent" antigen, formed from homologous protein, will give rise to formation of antibody. On the basis of a "contact" theory of antibody formation such as the Haurowitz-Mudd theory, discussed by Burnet (1941), a molecule of antigen containing only one determinant group, would be unable to effect its "imprint" on more than one region of the antibody protein in the course of synthesis, and a univalent or incomplete antibody molecule would be the result.

This line of thought therefore leads us to ascribe the presence of unusual quantities of incomplete antibody in some of our sera at least in part to the heterogeneous nature of the effective antigen. When immunization is effected in the ordinary way by injection of a foreign protein or of an artificial protein conjugate prepared with the usual precautions to maintain homogeneity, the antigenic molecules will be relatively uniform in hapten content. In our experiments on the contrary, for reasons which we have given, and particularly in those cases in which the formation of incomplete antibody is most marked, the antigenic stimulus is in all probability due to a range of antigenic molecules containing from one hapten group per molecule upwards. The heterogeneity of the antigen formed *in vivo* will be further increased by the fact that the pro-antigen may react, not with one only, but with many different body proteins.

There is one further factor in our experiments with possible bearing on the process of antibody formation which should be mentioned; this is the possible effect of the presence of the hydrolysed pro-antigen on the antigenicity of the conjugate formed *in vivo*, under the special conditions of our experiments in which its formation is contemporaneous with formation of the antibody itself, and possibly occurs at the site of production of antibody. This could have no more than a transient influence, however, owing to the solubility and diffusibility of most of the hydrolysis products at body pH.

None of these complicating factors, however, affects the validity of the main conclusion to which the present work has led, namely that simple substances of the type we have studied exhibit pro-antigenic activity not only in respect of skin sensitizing and anaphylactic antibody production, but in stimulating the production of circulating antibodies. Such pro-antigenic activity is doubtless a general property of compounds which share the one essential feature of a functional group capable of reacting with protein in the conditions which obtain in the cells and tissue fluid of the body; it may be a property also of compounds which, in the course of their metabolism, form products containing such a functional group.

SUMMARY.

1. A series of compounds, sharing in common the power of combining with proteins, and likely by so doing to introduce a new immunological specificity, has been examined for their power to induce antibody formation.

2. All the compounds studied have produced skin sensitivity and/or the anaphylactic state in guinea-pigs, when injected intradermally.

3. All the compounds have also, when injected intraperitoneally into rabbits,

given rise to the formation of specific precipitins, in some cases in high titre, which are detectable by *in vitro* reaction with a conjugate with gelatin of the compound employed.

4. The power of these compounds to induce antibody formation is designated pro-antigenic, since the actual stimulus to the antibody-forming mechanism is probably a full protein antigen produced by their interaction with body protein.

5. Semi-quantitative study of the serological reactions has enabled tentative conclusions to be drawn as to the influence of the chemical structure and reactivity of pro-antigens on their efficiency in inducing antibody formation.

6. The results help to bridge the gap which has so far existed between the action of typical skin-sensitizing compounds in causing formation of "fixed" antibodies, and the action of the same compounds when artificially linked to a protein in eliciting circulating antibody formation.

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NON-AGGLUTINATING ANTIBODY IN HUMAN ANTISERA TO *SH. SHIGA* AND *S. TYPHI*.

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FROM time to time observations on the specific immunological properties of anti-bacterial sera, human and animal, have been difficult to explain on the basis of the generally accepted conceptions of immunology. For example, sera are described which must contain significant amounts of immune-body as judged by the results of protection tests employing infection with virulent organisms, but which fail to show the presence of specific agglutinins or precipitins (Avery and Goebel, 1933; Chow, 1936; and Topley, 1937), and a possible explanation is to be found in the recognition of an incomplete, univalent or immature form of antibody, as it has been variously called, and indeed, Heidelberger, Treffers and

Mayer, 1940; Pappenheimer, 1940; Pappenheimer, Lundgren and Williams, 1940, have obtained some evidence, largely on the basis of precipitation reactions, for the existence of such incomplete antibody.

Immune sera with properties very similar to those described in these papers have been produced artificially and have recently been studied in considerable detail. For example, the conversion of antibody to the non-agglutinating and non-precipitating form has been achieved by the action of heat (Kleczkowski, 1941*a*, *b*, 1943; Bawden and Kleczkowski, 1942; Hooker and Boyd, 1942), by treatment with diazo-compounds (Eagle, Smith and Vickers, 1936; Heidelberger and Kabat, 1937) and formaldehyde (Eagle, 1938), and the early observations of Fleischmann (1905) that the precipitating or agglutinating power of immune sera could be destroyed by photodynamic irradiation without coagulation of the serum proteins has been confirmed and extended by Ross (1938), Zia, Chow and T'ung (1938), Smetana (1938), Smetana and Schemin (1941), Tyler (1945*a*, *b*), and Tyler and Swingle (1945), who have shown that photo-oxidized immune sera are able to combine with the homologous antigen, and that there appears to be little change in the specific nature of the combination. On the basis of the generally accepted theory of agglutination and precipitation put forward by Marrack (1938) and Heidelberger (1939), the destruction of the capacity of artificially modified antibodies to give precipitation or agglutination with the homologous antigen can be explained most simply as being due to the conversion of a multivalent antibody molecule into a univalent one.

Another method of demonstrating the existence of incomplete antibody in a serum is by means of its power of interfering with the reactions of a second homologous but normally reacting serum. Coca and Kelly (1921), working with a number of *H. influenzae* strains and their respective anti-sera, were able to do this, showing that quite small non-agglutinating amounts of one particular serum inhibited specific agglutination by another potent serum added subsequently. Jones (1928) likewise demonstrated the inhibition phenomenon, using *S. choleraesuis* and *Br. abortus* sera.

Recently the presence of specific immune-body which combines with erythrocytes but is unable to agglutinate them has been revealed by Race (1944) and Wiener (1944), and Coombs, Mourant and Race (1945) have elaborated a technique for the detection and measurement of immune-body possessing this characteristic property. The method consists in exposing the erythrocytes to the serum under investigation whereby any specific immune-body present combines with the homologous antigen on the cell surface. If the immune-body is of the normal type the erythrocytes will be agglutinated. If, however, incomplete antibody is present alone, although bound on the cell surface it will be unable to bring about agglutination. The erythrocytes are recovered by centrifugation, washed with saline to remove unadsorbed proteins and resuspended in saline. On the assumption that the cells have retained specifically the incomplete isoagglutinins present in the serum, and that these are globulins, a rabbit anti-human γ-globulin immune serum is added. This reacts with the sensitized cells and gives rise to agglutination. It is of interest to recall that so long ago as 1908 Moreschi used a similar series of reactions. He described a goat anti-rabbit red cell serum which sensitized but did not agglutinate rabbit erythrocytes. The fixation of the goat anti-rabbit serum on the rabbit erythrocytes was detected by the agglutination brought about on the addition of a rabbit anti-goat serum to the washed

and sensitized rabbit erythrocytes. Moreschi (1908) speculated as to whether something similar might not hold for bacteria and antibacterial sera.

In an attempt to establish, by the use of the technique described by Coombs, Mourant and Race (1945), the presence of incomplete bacterial antibody, we have examined the sera of individuals after immunization with the O-somatic antigen of *Sh. shiga* or with typhoid vaccine (T.A.B.C.). In some instances the normal sera taken before any previous association with either micro-organism were included for examination.

EXPERIMENTAL METHODS.

Anti-*Sh. shiga* immunization was carried out as described by Morgan and Schütze (1943). Two 0·1 mg. doses of phenol extracted somatic antigen (Morgan and Partridge, 1942) were given with an interval of 6 weeks. Four persons received one dose only of 0·1 mg.; all were bled 8 days after the last dose. For anti-typhoid immunization the customary two doses of alcohol-killed T.A.B.C. vaccine were given with an interval of three weeks.

The cultures used for agglutination and sensitization were *Sh. shigae* 4837 of the National Collection of Type Cultures, a hypo-agglutinable strain (Schütze, 1944), and *S. typhi*, Bhatnagar, Vi 1, a strain which fails to react significantly with H and O antibodies.

Human γ-globulin was prepared as described by Kekwick (1940).

Rabbit anti-human γ-globulin was prepared by giving intravenously to rabbits six doses, each of 1 mg., of human γ-globulin at about 4-day intervals. The animals were bled 7–9 days after the last dose. The presence of immune-body in the serum was established by precipitation tests using the human γ-globulin as test antigen.

The estimation of antibody by "γ-agglutination."

To 1·2 ml. amounts of a series of saline dilutions of the human serum under examination was added 1·2 ml. of saline suspension (Brown's Opacity 1) of *Sh. shigae* or *S. typhi*. The mixtures were incubated at 37° for one hour, centrifuged, and the bacteria washed twice in 4 ml. saline. After final washing the deposit was resuspended in 1·2 ml. saline, thus restoring the original opacity of the bacterial suspension, now sensitized with the test serum. From each tube 0·5 ml. of the suspension was transferred to 0·5 ml. of rabbit anti-human γ-globulin serum diluted 1 : 40, and a further 0·5 ml. of the suspension to an equal amount of a similar dilution of normal rabbit serum. The row of tubes containing normal rabbit serum serves to control the specificity of the γ-agglutination of the sensitized culture.

After standing at 37° for 2 hours and overnight at room temperature the agglutination results are read with the aid of a lens. Preliminary controls established the fact that the washing of sensitized bacteria in the manner described in no way altered their agglutinability, and that any agglutination occurring in dilutions beyond the normal titre limit would not be due to the repeated centrifugation.

A typical result of such a γ-agglutination experiment with anti-Shiga serum " 17 " is shown in Table I, where it will be seen that while the ordinary agglutination titre is 25, the γ-agglutination titre reaches 400, representing a 16-fold amplification. The γ-agglutination control titre does not exceed that of the

TABLE I.—*Ordinary and γ-Agglutination Titres for Anti-Shiga Serum "Ki."*

Serum dilutions.	1:6	1:12	1:25	1:50	1:100	1:200	1:400	1:800	1:1600
Ordinary Agglutination	++	++	+	−	−	−	−	−	−
γ-Agglutination after sensitization	++	++	++	++	++	++	++	+	−
γ-agglutination controls	++	++	+	−	−	−	−	−	−

ordinary agglutination. That the greatly increased γ-agglutination titre is not due to the non-specific adsorption of γ-globulin is demonstrated by the fact that if *S. typhi* is substituted for *Sh. shigae* the γ-agglutination titre now only equals 25, which, as will be seen later, is often found to be the typhoid Vi γ-agglutination titre of a normal serum. Similar evidence for the specific nature of the reaction is obtained by further cross-sensitization using an anti-typhoid serum "B." Here, with a suspension of *S. typhi*, the homologous γ-agglutination titre is 400, whereas with *Sh. shigae* suspension it is only 32—a typical Shiga γ-agglutination titre for a non-Shiga serum.

In Table II are grouped the agglutination titres of a series of sera obtained from normal and immunized individuals, the latter specimens being collected 8 days after the second dose of Shiga O-somatic antigen. The titration results include the titres obtained by the usual method of direct titration against a living suspension of *Sh. shigae*, as well as the agglutination titres obtained by means of the rabbit anti-human γ-globulin serum. In each instance there is an amplification of titre by employment of the γ-agglutination technique, and there appears to be a somewhat greater increase among the immunized than among the non-immunized, for while the average amplification with normal sera is a six-fold one, with immune sera it is eighteen-fold.

TABLE II.—*Ordinary and γ-agglutination Titres Before and After Immunization with Shiga Somatic Antigen.*

Normal sera.	Agglutination. Ordinary.	Agglutination. γ-Globulin.	Amplification of titre.	Immune sera.	Agglutination. Ordinary.	Agglutination. γ-Globulin.	Amplification of titre.
1	12	25	2	14	100	400	4
2	50	200	4	3	128	512	4
3	16	64	4	15	100	800	8
4	6	50	8	16	50	400	8
5	25	200	8	9	32	256	8
6	50	400	8	10	128	1024	8
7	50	400	8	11	32	256	8
8	8	64	8	17	25	400	16
9	2	16	8	18	50	800	16
10	16	128	8	19	25	800	32
11	16	128	8	20	25	800	32
12	4	64	16	21	6	200	32
13	12	400	32	22	6	400	64

Average amplification: For normal sera, 6; for immune sera, 18. Individuals 3, 9, 10, 11 received only one dose of Shiga antigen; all others had two.

A similar series of titrations was carried out by the two methods on a number of sera obtained from normal and immunized persons who had received two doses of alcohol-killed T.A.B.C. vaccine. The serum was taken seven days after each of the two doses. Control tests again showed that normal typhoid Vi agglutination was in no way disturbed by centrifugation and washing, so that these procedures could not be held responsible for any change in titre observed. Table III records the titres obtained. Here again in each serum tested the γ-agglu-

TABLE III.—*Ordinary and γ-Agglutination Titres with Typhoid Vi Before and After Immunization with* S. typhi.

Sera.	Agglutination titre before immunization.		Titre amplification.	Agglutination titre 7 days after 1st inoculation.		Titre amplification.	Agglutination titre 7 days after 2nd inoculation.		Titre amplification.
	Ordinary.	γ-.		Ordinary.	γ-.		Ordinary.	γ-.	
A	3	12	4	12	200	16	12	200	16
B	3	12	4	12	400	32	12	200	16
C	<2	12	–	<2	25	–	6	100	16
D	<2	25	–	12	400	32	25	400	16
E	6	25	4	25	400	16	25	200	8
F	12	50	4	25	400	16	25	200	8

Average amplication: For normal sera, 4; for immune sera 22 and 13.

tination titre exceeds that recorded by ordinary agglutination methods; the average amplification for the sera of non-immunized persons was 4-fold; for the corresponding sera of immunized persons it was 22 and 13-fold respectively for the two bleedings. Once again a greater increase is obtained for immune than for normal sera, but it would seem as if this increased ratio is not maintained, for an examination of sera obtained from seven persons whose immunization with alcohol-killed T.A.B.C. vaccine had taken place two to three years previously gave 2, 2, 2, 4, 4, 4, and 16-fold amplifications in titre. Only in the last individual, and the same result was obtained from two separate bleedings that were made at a few days' interval, did the ratio reach that of the sera of recently immunized persons.

Heat Resistance of the Non-agglutinating Antibody.

Coombs, Mourant and Race (1945) have demonstrated that heating sera at 65°–70° for 5–10 minutes does not destroy the capacity of incomplete Rh agglutinins to unite with erythrocytes and subsequently with anti-γ-globulin serum, whereas complete antibody loses its agglutinating properties after heat treatment. The results given in Table IV confirm this finding for the non-agglutinating antibody in an anti-"Shiga" serum.

TABLE IV.—*Heat Resistance of Ordinary Agglutinating and Non-agglutinating Antibody.*

Anti-Shiga serum × *Sh. shiga.*		1:6	1:12	1:25	1:50	1:100	1:200	1:400	1:800	1:1600
Serum unheated	Ordinary agglutination	+++	+++	++	++	–	–	–	–	–
	γ-Agglutination	+++	+++	++	++	++	++	+	⊥	–
	γ-Controls	+++	+++	++	++	–	–	–	–	–
Serum heated undiluted at 65° C. for 20 mins.	Ordinary agglutination	–	–	–	–	–	–	–	–	–
	γ-Agglutination	++	++	++	++	++	+	–	–	–
	γ-Controls	–	–	–	–	–	–	–	–	–

Anti-typhoid serum × *S. typhi* Vi.		1:6	1:12	1:25	1:50	1:100	1:200	1:400	1:800	1:1600
Serum unheated	Ordinary agglutination	+++	++	+	–	–	–	–	–	–
	γ-Agglutination	+++	++	++	++	++	+	⊥	–	–
	γ-Controls	+++	++	+	–	–	–	–	–	–
Serum heated undiluted at 65° C. for 20 mins.	Ordinary agglutination	+++	++	+	–	–	–	–	–	–
	γ-Agglutination	+++	++	++	++	++	+	⊥	–	–
	γ-Controls	+++	++	+	–	–	–	–	–	–

Heating at 65° does not destroy this antibody; it still sensitizes, and the adsorbed γ-globulin is still agglutinable by subsequently added anti-γ-globulin serum, although the γ-agglutination titre is somewhat diminished. Ordinary Shiga antibody, on the other hand, loses its agglutinating powers.

In the case of anti-typhoid serum neither ordinary nor non-agglutinating Vi antibody suffers from such heating.

Blocking Experiments.

The demonstration of specific antibody blocking powers in one abnormal non-agglutinating anti-influenza serum by Coca and Kelly (1921), and in *S. cholera-suis* and *Br. abortus* sera rendered non-agglutinable by heat by Jones (1928), led us to consider the possibility of demonstrating similar blocking by the anti-typhoid and anti-Shiga sera under investigation.

Sera with high non-agglutinating antibody titres were chosen, and used in as high concentration as was possible without bringing about ordinary agglutination. After sensitization with such sera, and in parallel with non-immune sera, the suspensions were centrifuged, resuspended in saline and compared with unsensitized suspensions in an ordinary agglutination test. In no case was there any indication that sensitization with the non-agglutinating antibody interfered with ordinary agglutination, and this was so whether the agglutinating serum was a human serum like the sensitizing one or one prepared in the rabbit.

As, however, the sera under consideration here contain relatively high titres of complete antibody and can be used in the blocking test only in dilutions above that level, interference with ordinary agglutination might not be expected. Reference to Table II in Tyler's (1945*a*) paper shows that serum dilutions corresponding to those that could be used with our sera produced little or no blocking; Tables III and IV in Coca and Kelly's (1921) paper show similar results.

An attempt was therefore made by repeated exposure of bacterial suspensions to a serum dilution that was above the ordinary agglutinating titre and yet within the γ-agglutination level to demonstrate a cumulative effect of either incomplete or complete antibody, whichever of the two might be present. If it was incomplete antibody that was giving rise to γ-agglutination, then a repeatedly exposed suspension might finally accumulate sufficient incomplete antibody to show blocking, while, if it was small amounts of complete antibody that caused the γ-agglutination, the repeated exposure should ultimately result in ordinary agglutination.

TABLE V.—*Attempts to Block Ordinary Agglutination After Repeated Exposure of the Agglutinating Suspension to a γ-Agglutinating serum.*

Agglutinating serum.	Typhoid Vi suspension, treated and untreated.		Dilutions of agglutinating serum.					
			1 : 25	1 : 50	1 : 100	1 : 200	1 : 400	Control.
Typhoid case serum 342	Untreated		++	++	−	−	−	−
	Exposed 5 times to	Saline	++	++	⊥	−	−	−
		Serum H*	++	++	⊥	−	−	−
		Serum D†	++	++	⊥	⊥	−	−
			1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	Control.
Oxford standard typhoid Vi serum	Untreated		++	++	++	⊥	−	−
	Exposed 10 times to	Saline	++	++	++	+	−	−
		Serum H*	++	++	++	⊥	−	−
		Serum D†	++	++	++	++	⊥	−

* No γ-agglutination titre at the dilution employed, i.e. 1 : 70.
† Contains γ-agglutinating bodies at the dilution employed, i.e. 1 : 70.

In Table V the results of two such experiments are given. In each the Vi typhoid suspension was exposed to a 1 : 70 dilution of an anti-typhoid serum "D," whose ordinary Vi titre was 25 and whose γ-agglutination titre was 400. Each exposure consisted of 20 or more minutes in a water bath at 37°, followed by centrifuging and resuspension in 1 : 70 serum. Finally the suspension thus treated was tested for agglutinability against an anti-typhoid Vi serum, in the one case a human case serum, in the other Oxford Standard Serum.

There is certainly no evidence that incomplete antibody has been accumulated by the bacteria ; no interference with ordinary agglutination by the test sera is observed. Nor have even 10 exposures succeeded in supplying sufficient complete antibody to agglutinate the suspension ; the saline control remains unagglutinated, although a slight increase in the titre of the test serum is evident in each case as compared with the titre obtained when using (*a*) untreated suspensions, (*b*) suspensions exposed to a serum "H" (with no γ-agglutination titre when similarly diluted 1 : 70), or (*c*) to saline alone.

Non-agglutinating Antibody in Rabbit Sera.

By the use of fowl anti-rabbit precipitating serum for the γ-agglutination test, it was possible to examine rabbit sera for the presence of non-agglutinating antibody. In the normal and anti-Shiga sera tested these antibodies were found to exist in amounts sufficient to amplify the titre in some cases as much as 32 times.

DISCUSSION.

By employment of the γ-agglutination technique it has been possible to demonstrate an enhanced titre for every serum, normal or immune, examined. It remains to decide what this increase in titre implies. Does it necessarily follow that some form of incomplete antibody is the cause, or might not the amplified titre be due to small amounts of complete antibody present in the higher serum dilutions—amounts too small to occasion normal agglutination, but sufficient to sensitize the bacterial cell to anti-γ-globulin immune-body and thus bring about agglutination ?

Two tests might be expected to help decide this point. On the one hand, Witebsky, Mohn, Howles and Ward (1946) by dialysis were able to separate complete from incomplete antibody, and Tyler (1945*b*) by fractional precipitation with ammonium sulphate also accomplished a separation of the two types of immune-body. On the other hand, the blocking of ordinary agglutination has been used by various experimenters to demonstrate the presence of incomplete antibody. Neither of these methods, however, proved helpful in our experiments. Both methods of serum fractionation were carried out, but did not result in a separation of the two agglutination types and, as has been described, blocking of ordinary agglutination did not follow preliminary exposure to a purely γ-agglutinating serum dilution, possibly because the dilutions in which the sera could be used were too high. Repeated exposures to the serum dilution, moreover, did not circumvent this difficulty.

Although it has not been possible to carry out a blocking experiment with the antibodies responsible for the γ-agglutination titre, and fractionation has not succeeded in separating them from the ordinary complete antibody, their existence

as a separate entity seems indicated by the manner in which this titre relative to that of the complete antibody varies, sometimes being so little as twice, at others as much as 64 times as high ; also a greater preponderance of non-agglutinating antibody relative to agglutinating antibody has been noted in the sera of recently immunized individuals.

SUMMARY.

The presence of an antibody which, while not capable of agglutinating its specific organism, unites with the bacterial cell and then reacts with anti-γ-globulin serum, has been observed in a series of normal and anti-Shiga and anti-typhoid human sera. The titre of such antibodies may be as much as 64 times that of the corresponding ordinary agglutination titre. Such amplification of titre is usually greater in the immune than in the non-immune serum.

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STUDIES ON EXPERIMENTAL GOITRE. VII: THYROID CARCINOMATA IN RATS TREATED WITH THIOUREA.

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THE production of thyroid neoplasms by the long continued action of the goitrogenic agent of rape seed was reported by Griesbach, Kennedy and Purves (1945). The present paper describes some of the results observed after long term administration of the goitrogenic substance thiourea. As might be expected from the results of rape seed administration, thyroid tumours were produced in most of the animals receiving thiourea. In two rats out of thirty studied, metastases of thyroid origin were found histologically in the lungs. This communication describes the appearances observed in these two animals.

MATERIALS AND METHODS.

The rats were of the local Wistar strain which was used in previous experiments with rape seed diet. They were maintained on the diet S 3 described previously (Griesbach, Kennedy and Purves, 1945). From the age of eight weeks they received thiourea (0·25 per cent in the drinking water).

RESULTS.

Animal 1.

This animal after twenty-two months of thiourea treatment was observed to be in poor condition and was rapidly losing weight. It was therefore killed. At the time of death it weighed 90 g. (average weight of the group 140 g.). The thyroid was approximately 150 mg. in weight. The enlargement of the thyroid was more marked on the right side. The surface was smooth, and there was no adherence to the adjacent tissues. On histological examination the right lobe was found to be almost entirely replaced by a thyroid neoplasm. The neoplasm was not sharply defined, but seemed to be invading the normal thyroid tissue. Throughout the neoplasm there were large accumulations of colloid which created colloid lakes or cysts, while between these cysts there were highly cellular areas in which the growth was papillary. Invasion of the wall of the thyroid veins at the hilus had occurred, and the neoplastic tissue was in places in direct contact with the blood stream. As shown in Fig. 2, fragmentation of the growth with detached fragments floating in the blood stream was observed. The normal thyroid tissue was less activated than is usual for animals receiving thiourea. This result was probably consequent on the cachexia and malnutrition from which the animal was suffering, as under these conditions the thyrotropic secretion of the pituitary is inhibited. The colloid accumulation and relatively inactive epithelium of the neoplasm are probably due to the same effect, since we have found that the thyroid neoplasms

produced by thiourea are profoundly modified by variations in the level of thyrotropic hormone secretion.

No signs of metastases were observed on naked-eye examination at the autopsy of the animal. Histological examination of the lung revealed numerous metastases of typical thyroid structure. Well-defined acini containing deeply staining colloid were present. Most of these metastases were in close relation to branches of the pulmonary artery. In some cases the growth occupied the whole lumen of the artery, which could only be identified with certainty by examiinng serial sections. In some of these metastases the growth inside the blood vessel was associated with a spreading invasive growth which had penetrated the vessel walls and infiltrated the adjacent lung tissue. No metastases were observed in sections of the liver or kidney. The bones were not examined.

Animal 2.

This rat, after twenty-three months of thiourea administration, was treated with injections of a thyroid powder brought into solution by peptic digestion. These injections supplied ten micrograms of thyroid iodine per day, and were given to suppress the secretion of thyrotropin by the pituitary, and so promote colloid storage in the thyroid neoplasm should this be still under control of the thyrotropic hormone. After three weeks of thyroid injections the animal was killed. Its weight was 190 g. and the thyroid was 134 mg. The left lobe was markedly larger than the right. The surface was smooth, and there was no adherence to surrounding tissues. Histologically the larger lobe of the thyroid was almost entirely replaced by a neoplasm. In this there was some degree of colloid accumulation but this was not marked, and the acinar spaces were irregular or slit-like in section with folding of the epithelium. The cells were not flattened, and the appearance was much more active than that seen in benign thyroid neoplasms after thyroid treatment. Invasion of the wall of the large veins had occurred. In one place a fragment of thyroid growth lying free in the lumen of a thyroid vein was seen (Fig. 6). This thyroid neoplasm contained in one place a mass of solid cellular growth of different structure from the main tissue of the neoplasm. This mass was considered to be of the same nature as the foetal adenoma of human pathology. In the other thyroid lobe there was a single small adenoma quite benign in appearance.

At autopsy no signs of metastases were observed by naked eye examination. Histologically the lungs were found to contain multiple metastases similar to those seen in animal 1. Well defined acini were present, and colloid accumulation was general and much more marked than in the primary growth in the thyroid. The cells, however, though involuted to some extent, were not anywhere markedly flattened. Relationship to the branches of the pulmonary artery were as clearly marked in this animal as in the first, and will be clearly seen in Fig. 7. No metastases were found in sections of the liver or kidney. The bones have been reserved for histological study.

DISCUSSION.

Bielschowsky (1944) reported the production of thyroid tumours in rats by the combined actions of the carcinogenic substance, 2-acetamido-fluorene and the goitrogenic substance, allyl-thiourea. Griesbach, Kennedy and Purves (1945)

reported the production of thyroid tumours by the action of the goitrogenic agent in rape seed, which showed that the carcinogenic agent was unnecessary for the induction of thyroid tumours. Bielschowsky (1945) obtained thyroid tumours by the action of allyl-thiourea alone, but considered that the thyroid tumours induced by the goitrogenic agents acting alone were all benign as compared with those appearing after treatment with carcinogen in combination with goitrogenic agents. This opinion was based on the histological appearance of the primary growths, as metastases were not observed by Bielschowsky in any of his rats.

The results reported in this paper show that the thyroid tumours induced by goitrogenic agent alone (in this case thiourea) vary in their degree of malignancy, and that some of them if given time demonstrate their malignant behaviour by the production of metastases. The comparatively long time (two years approximately) for the development of these metastases in the rat is consistent with the view that only a minor degree of malignancy exists in these growths. It does not appear that any malignant change has supervened in a previously benign adenoma, because no nodule of carcinomatous structure was found in association with an adenoma of benign histological appearance. However, the nodule of solid cellular growth which was found in one primary thyroid tumour in rat No. 2 has evidently arisen by secondary change in the pre-existing adenocarcinoma. In our opinion this structure corresponds to the growth which has been called " foetal adenoma " in human pathology. This name, however, only means that there is little or no attempt at acini formation, so that the structure resembles that of the foetal thyroid at an early stage in its development. The cells of the foetal adenoma stain much less intensely both in the cytoplasm and in the nucleus than the cells of the carcinoma. None of the metastases found resemble the " foetal adenoma " structure, so that its classification as a benign neoplasm seems to hold for the rat as well as for man.

In rat No. 2, the treatment with thyroxine which inhibits the thyrotropin secretion of the pituitary has produced an involuted type of epithelium compared with the appearance of other thiourea induced tumours which we have seen, and

DESCRIPTION OF PLATES.

FIG. 1.—Thyroid gland of Rat No. 1, showing adenocarcinoma. Colloid accumulation and areas of papillary growth are shown. The wall of the vein to the right of the figure is extensively invaded by the tumour. H. and E. × 90.

FIG. 2.—Another field of the section shown in Fig. 1, showing tumour cells in contact with the blood stream. H. and E. × 400.

FIG. 3.—Metastasis in lung of Rat No. 1. The central area containing large acini is surrounded by an area characterized by imperfect acini formation. H. and E. × 140.

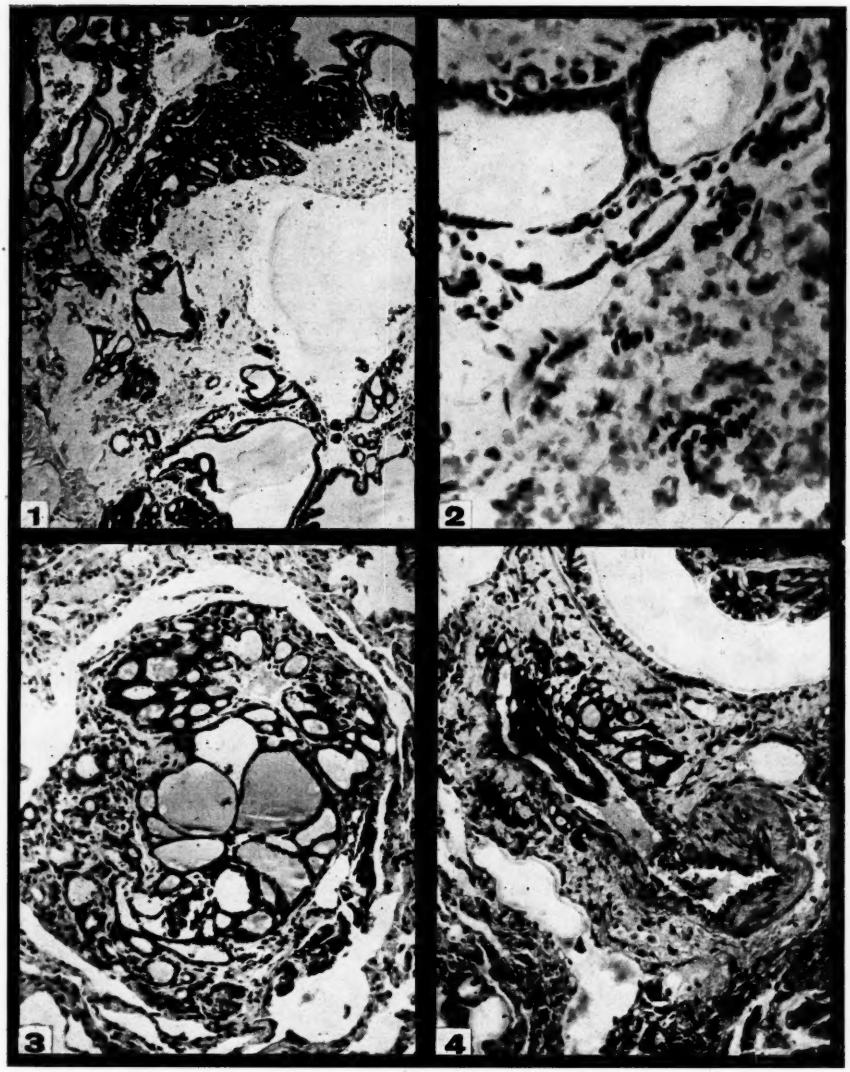
FIG. 4.—Lung of Rat No. 1. A metastasis is situated inside a branch of the pulmonary artery. An extension of this growth is infiltrating the tissues outside the artery. H. and E. × 140.

FIG. 5.—Thyroid gland of Rat No. 2, showing at the top of the figure some normal thyroid tissue. Below this is adenocarcinoma, and at lower right the edge of a nodule of foetal adenoma. H. and E. × 90.

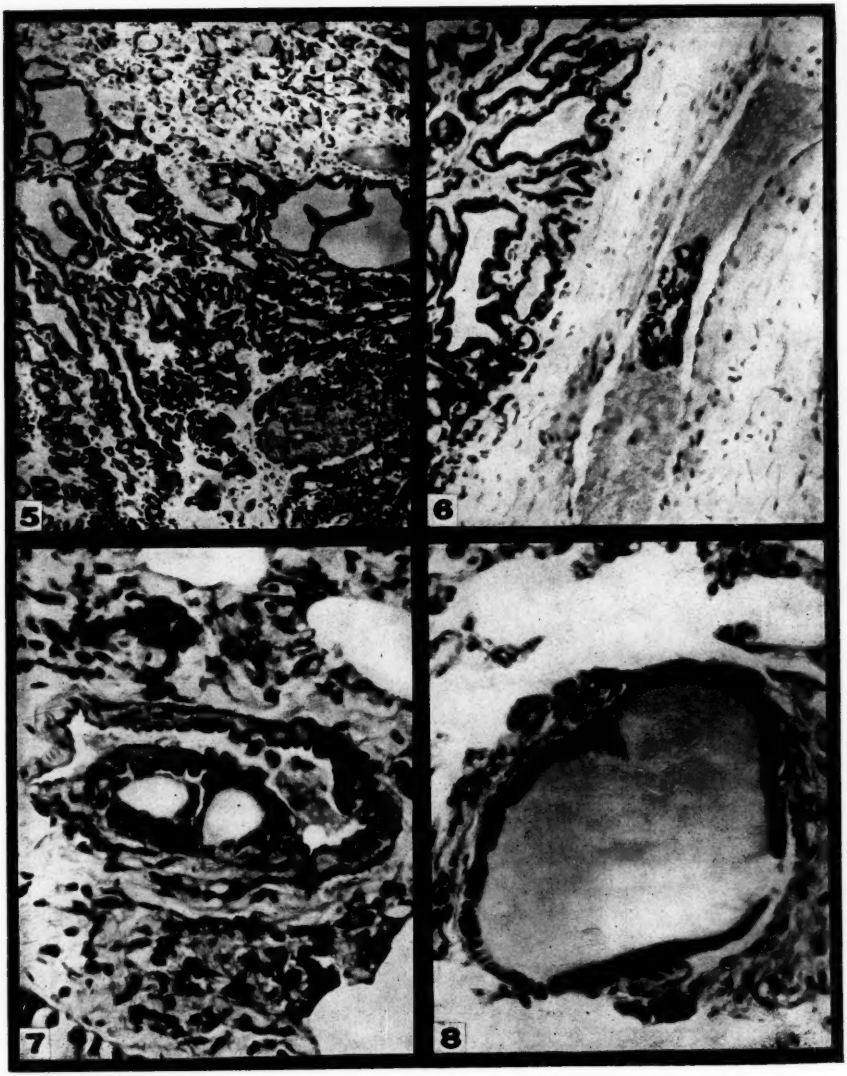
FIG. 6.—Thyroid gland of Rat No. 2, showing thyroid carcinoma on the left. In the centre is a vein containing carcinoma tissue in the lumen. H. and E. × 140.

FIG. 7.—Lung of Rat No. 2, showing a branch of the pulmonary artery in cross-section. The artery contains a small thyroid metastasis consisting of two acini containing colloid. The metastasis is covered with endothelium, and the remainder of the lumen is patent and contains blood. H. and E. × 650.

FIG. 8.—Lung of Rat No. 2, showing a thyroid metastasis consisting of a single acinus distended with colloid. H. and E. × 400.



Purves and Griesbach.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the involution of the metastases where colloid accumulation is conspicuous is quite evident. It seems, therefore, that these adenocarcinomas of low grade malignancy are still responsive to changes in the level of thyrotropic hormone. Judging from the histological appearances we would predict that thyroid medication if given earlier would have inhibited the development of these growths and their metastases, an effect which might have clinical application in certain cases of human thyroid malignancy. The marked tendency of the tumours here described to invade veins is characteristic of thyroid adenocarcinoma in human pathology, and the occurrence of masses of tumour cells lying within the blood vessels is frequently observed in human material. The occurrence of metastases in the lungs developing from tumour cell emboli in the branches of the pulmonary artery is therefore to be expected.

We believe that the production of these thyroid tumours is due, not to any direct carcinogenic action of the thiourea administered, but rather to the excessive and prolonged stimulation of the thyroid epithelium by the thyrotropic secretion of the pituitary. This action would be analogous to the specific production of breast tumours in susceptible animals by the administration of excessive amounts of oestrogen.

SUMMARY.

1. In thirty rats in which thyroid tumours had been induced by administration of thiourea for a period of nearly two years, two animals were found at autopsy to have malignant thyroid tumours.

2. The malignant nature of these thyroid tumours was evidenced by their invasion of the thyroid veins, and by the presence of numerous metastases in the lungs.

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TASTE AND SELECTION OF FOOD IN HYPOGLYCAEMIA.

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RICHTER and Campbell (1940), Richter (1942), Richter, Schmidt, jun., and Malone (1945) have shown that when experimental animals are allowed to select their diet from a series of foods, they choose in accordance with the constituents and concentrations in the body fluids; in other words, they try to maintain what Claude Bernard called a constant internal environment necessary for the maintenance of life. Richter has also shown that this self-regulatory activity depends on the sense of taste. The objections to applying the self-selection technique as used by Richter to man are obvious: conditioning by upbringing during the long infantile period; by regional feeding habits;

by the conventions of the table and by self-control, as well as by personal predilections, tend to impede self-regulation in human beings and make it less predictable. Richter *et al.* (1945) also speaks of man's defective taste mechanisms. In an emergency like hypoglycaemia, however, one would expect cultural habits and personal factors to be of little weight in comparison with the vital demands of the body.

The behaviour of patients undergoing insulin treatment, described Sakel (1938) as "hunger riot," seems to confirm that, at a certain stage of hypoglycaemia, schizophrenics break all rules and conventions in their overwhelming craving for carbohydrates. Hunger riot, however, far from being a regular feature among hypoglycaemic patients, is rather infrequent in the routine treatment of schizophrenia. Another common finding inconsistent with self-regulation is the diabetic who, when hypoglycaemic through an overdose of insulin, angrily refuses to take sugar offered by the people around him, since he lacks insight into his abnormal condition. While he still seems conscious and aware of his surroundings, his self-regulation is apparently deficient. The effect of hypoglycaemia on cortical function and consciousness has, of course, to be taken into account whenever self-regulation is tested in this condition. In view of these contradictory observations, it seemed worth while to approach the subject experimentally, using the self-selection technique with the object of finding out whether and, if so, how far self-regulation operates in the hypoglycaemic patient. If its operation was found to be the general rule, individual deviations from it may deserve closer analysis; the corresponding subjective taste sensations unknown in animal experiments would be worth while studying; and finally, one could try to interfere with self-regulation by using substances of deceptive taste or distractive smell.

The present paper is based on about 250 experiments arranged under the above propositions.

METHOD AND MATERIAL.

In the main series of tests the patient was asked to take small sips from five glasses of colourless liquids marked A, B, C, D and E, and to describe their taste. He was also asked which of the liquids he would like best for a long drink. If a subject was in doubt about his final preference, he was allowed to re-taste the whole series, or a couple of glasses, before making up his mind. Notes were made of the subject's reactions towards each liquid and of his final choice, and blood glucose determinations were made at the time, using the method of King, Haslewood and Delory (1937). In retesting the same individual care was taken that the order of the five solutions was not the same twice running; in fact, the order was varied on each testing day.

The five solutions used were as follows: water, 5 per cent sucrose, 30 per cent sucrose, 0·5 per cent sodium chloride, and saccharine. The saccharine solutions were made to equal, as nearly as possible, the sweetness of the 30 per cent sucrose solution. This was not altogether easy, since the sweetness of saccharine does not follow a simple rule, and becomes proportionately less sweet as the concentration increases. The low solubility of the substance and the varying degree of sweetness of various samples are additional complications. Each sample was made up immediately prior to use, and was standardized by

experimental tasting by the same experimenter, so that in each series saccharine sweetness was as nearly as possible identical with that of the 30 per cent sucrose solution. The temperature of all liquids was kept equal with that of tap water.

The subjects of the experiments were physically healthy individuals between the age of 17 and 45, undergoing Sakel's insulin therapy for early schizophrenia. It was considered unlikely that their mental illness would interfere with the experiments. Schizophrenics sometimes refuse food, others devour everything they can get hold of regardless of taste. Such patients were not included among our subjects, and cases of catatonic stupor and similar states, in which clearness of consciousness was doubtful, were also left out. Some peculiar predilections in our series may be put down to perversions of taste due to the mental condition. On the whole, however, it can be maintained that schizophrenia does not affect the sense of taste and that our patients' behaviour in these tests was not essentially different from that of normal subjects in hypoglycaemia.

The experiments were made in various stages of hypoglycaemia, covering all glucose levels from fasting to that of incipient hypoglycaemic coma. By proper timing of the experiment during the progressive hypoglycaemia we succeeded in obtaining a sufficient number of results even within the lower range of blood glucose. As long as the patient understood the instructions, could hold the glass, describe the taste and express his preference, his case was included even if he seemed slightly drowsy.

The following example may illustrate the procedure under such conditions.

Case R—, 19.5.44.

7.30 a.m. : 100 units insulin.

10.35 a.m. : Sweating, narrow pupils, but seems quite clear.

A. (saccharine) : "Good."

B. (NaCl) : "Too salty."

C. (5 per cent sucrose) : "Better than A."

D. (H_2O) : "No."

E. (30 per cent sucrose) : "I like E. Prefer it to A. C is probably the best—can I try again ?" Tastes C, E, and A.

"E, I think, is better than C or A."

Blood glucose 23 mg. per 100 ml.

RESULTS.

The main series of experiments consisted of 202 observations in 100 patients. The first result showed that the preference for the 30 per cent sucrose solution as a long drink was definitely related to the blood glucose level. Above a level of 50 mg./100 ml. the proportion of subjects preferring this solution was low, but below this level the proportions were reversed. In Figs. 1 and 2 illustrating this result the experiments are divided into groups according to the blood glucose level, each group covering a range of 5 mg. glucose per 100 ml. Three experiments where no decision could be obtained were omitted. Fig. 1, giving the absolute numbers of experiments, shows those preferring 30 per cent glucose above, those choosing one of the other liquids below the line. It conveys an idea of the number of experiments in each group. In Fig. 2, the same preferences are given in percentages of the group and plotted against the blood glucose group.

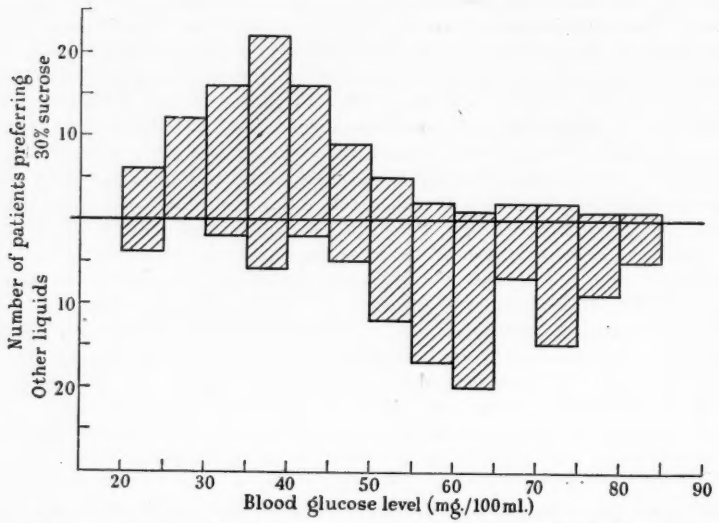

FIG. 1.—Preference (actual numbers) for 30 per cent sucrose in relationship to blood glucose level.

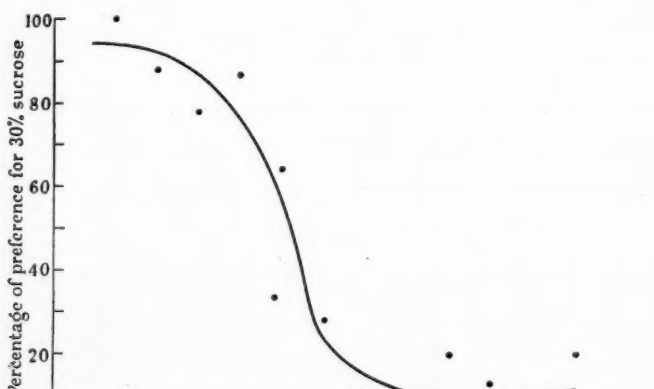

FIG. 2.—Percentage of Preference for 30 per cent sucrose in relationship to blood glucose level.

The great majority of subjects whose blood glucose level was over 50 mg. per 100 ml. rejected the 30 per cent sugar solution as too sweet, while only a small minority of those with blood glucose below this value preferred one of the other liquids to this solution.

The result leaves no doubt that the taste in hypoglycaemia is governed by the principle of relf-regulation and that the influence of conditioning factors is negligible. The change of taste was found to occur at almost one point of the blood glucose scale.

Having thus established the validity of the general principle of self-regulation in human hypoglycaemia, we can now consider the results in greater detail.

30 per cent sucrose solution.—This was preferred to other liquids on 95 occasions ; only in eleven of these tests, performed on nine subjects, was the blood glucose level above 50 mg./100 ml. Five of these results came from three patients who showed other abnormal reactions. They will be discussed later in the paragraph on saccharine. Subjects repudiating 30 per cent sucrose described it as " syrupy," " sweet and oily," awfully sweet," " very sweet syrup," etc.

5 per cent sucrose solution.—Many subjects recognized the similarity of the two sucrose solutions. 5 per cent sucrose was chosen as a long drink 28 times ; the blood glucose was 19 times above the critical level. In instances below this level its taste was described either as " mild or weak sweetness," " water with a wee bit of sugar," or as " watery," " plain," " rather dull," or " not much taste."

Omitting equivocal replies, 175 judgments on this solution were grouped according to the three criteria " sweet," " slightly sweet " and " tasteless." The percentage of " tasteless " was significantly higher in cases of blood glucose below 50 than in cases above this level (36 against 18 per cent), while " sweet " was higher above the critical level (19 against 32 per cent).

Water.—There is considerable loss of water during hypoglycaemia. Sweating is often the first sign of the vagotonic effect of insulin, followed by excessive secretions of saliva and of gastric juice ; bronchial and lacrymal secretions are also increased. Complaints of thirst are frequent. Water was, in fact, preferred to the others and was chosen as a long drink in approximately one quarter of the experiments (51). In only nine instances was the blood glucose below 50 mg. per 100 ml. Water was recognized in the majority of cases or described as tasteless.

NaCl solution.—Because of the simultaneous loss of chlorides by perspiration and gastric secretion, preference was expected for the 0·5 per cent salt solution. Only on one occasion, however, was it chosen as a long drink with a blood glucose of 70 mg. per 100 ml. The subject described its taste as " bitter " and at the same test the taste of water " like soda." Perversion of taste was suspected but it was found that the same subject had chosen 30 per cent sucrose when his blood glucose was 28 ; he then called the taste as salt " terrible." Aversion towards the salt solution was widespread. Disgusted grimaces, expressions like horrible, awful, unpleasant, were most frequent in the lower range of blood glucose. The description " bitter " occurred 16 times.

However, in 138 out of 199 experiments (three subjects were unable to give any description) the solution was correctly described as salt, salty, brine, etc. Only one patient maintained on two occasions that he liked the salt solution, although this did not influence his final choice which followed the general rule.

Saccharine.—Saccharine was included in the series of liquids for the following

reasons: The compounds which give rise to a sweet taste may be divided into two classes, the first of which contains a relatively large number of free hydroxyl groups, polyhydric alcohols and carbohydrates, e.g. glycerol and sucrose. The degree of sweetness is, to some extent, related to their food value. The second class consists of benzene derivatives in which the degree of sweetness is not related to nutritional value; an example of this class is saccharine. One of the problems of self-regulation is whether the hypoglycaemic subject will be able to distinguish between the "natural" sweetness of the first class of compounds and the "artificial" sweetness of the saccharine group.

In 24 cases saccharine was chosen as a long drink and preferred to the other liquids. Only three of these had, however, at the time a blood glucose level below 50 mg./100 ml. This seemed remarkable, considering that the sweetness of the saccharine was intended to be as close as possible to that of 30 per cent sucrose. In other words, self-regulation could not be deceived by the sweet taste of saccharine, a substance unable to raise the lowered blood glucose level.

It seemed of interest to group the instances in which saccharine was not recognized as sweet according to the blood glucose levels. Out of 45 cases denying sweetness of saccharine 33 had a blood glucose below 50 mg./per 100 ml. This can be interpreted as protection against the "artificial" sweetness where it is most needed. On the other hand, in 157 tests (78 per cent of the total) saccharine was recognized as tasting sweet, sweetish, sugary, too or slightly sweet, in 120 of these without further comment. A relatively small number of subjects (22) took notice of the admixture of the different taste saccharine has for most people, with expressions as follows: "sweet, but something else," "sweet and bitter," "sugar and salt." The verdict "bitter" occurred 13 times, salty only twice. Two subjects described the taste as "synthetic sweetness" and two others recognized the taste as that of saccharine.

Although only preferred to the other liquids in 12 per cent of the total, the majority of the subjects did not loathe saccharine. Unsolicited dislike was voiced 16 times, but 28 times the taste was spontaneously described as pleasant.

Detailed analysis of the 24 experiments, in which 18 subjects preferred saccharine to the other liquids, revealed that six subjects selected saccharine on two occasions; when two of these subjects were given one or more further tests their preferences seemed to follow the general trend. The same holds true of five subjects, who selected saccharine only once, but made their choice conforming to standard on earlier or later occasions. In three subjects, however, some perversion of taste could be suspected; they preferred saccharine at a blood glucose level of 49, 53 and 55; in repeated tests the same subjects selected the concentrated 30 per cent sucrose solution at even higher blood glucose levels, two of them on two different occasions (see paragraph on 30 per cent sucrose). Here a personal factor seemed to interfere with the self-regulatory mechanism; but no connection with the schizophrenic psychosis could be discovered.

To sum up, saccharine did not interfere with the prevailing principle of self-regulation, and the predilection for the sweet taste did not become unselective with the lowering of consciousness through hypoglycaemia.

Odour.—Some preliminary experiments were designed to test the effect of odour on self-regulation. They consisted of two series undertaken in the same subjects.

In the first part a series of aromatic extracts (peppermint, "strawberry," aniseed, cinnamon and clove), made up in 2·5 per cent sucrose solution, were presented to subjects in a fasting condition. The reaction to each solution and an expression of preference was obtained, and at the same time a blood glucose determination was made. Later the same subjects were re-tested in a condition of hypoglycaemia (blood glucose below 50). This time the five original solutions (5 per cent sucrose, 30 per cent sucrose, saccharine, water and 0·5 per cent sodium chloride) were used, but to all, with the exception of that containing 30 per cent sucrose, were added the aromatic substance for which a preference had been expressed in the first test. The subjects' reactions to the five solutions, the expression of preference and the blood glucose level were duly noted.

The results showed that in many cases the subject had a strong liking for one or other of the aromatic "flavours" when the blood glucose was normal; but that when in a state of hypoglycaemia the universal preference was for 30 per cent sucrose solution, despite the fact that this solution alone did not contain the preferred flavour.

DISCUSSION.

In an earlier paper one of the authors (Mayer-Gross, 1941) put forward the hypothesis that the spontaneous movements of the mouth and face in hypoglycaemia are produced by excitation of areas in the central nervous system connected with food intake. Deprived of carbohydrates, the principal fuel, the remaining energy of the central nervous system is concentrated on parts which normally subserve the removal of such an emergency.

The present experiments were carried out in an earlier stage of hypoglycaemia before oral and facial movements had made their appearance. They confirm the predominant craving for carbohydrates. At one point of the blood glucose scale this becomes so great that the normal dislike for the excessive sweetness of a 30 per cent sucrose solution is reversed into a preference for this liquid. The hypoglycaemic subject not only chooses the solution best suited to replace the glucose deficit, but also discriminates between two compounds of equal sweetness, where one is able to replace the loss of glucose and the other unable to do so. The discrimination is sharpest where the need is greatest. Loss of water and of chlorides due to hypoglycaemia have no influence on the subject's choice, which is solely governed by the emergency of the carbohydrate metabolism. Odour has no effect on the subject's preference.

These findings are well in agreement with the theory of self-regulation, which explains the purpose underlying the subject's behaviour.

The limitations of a theory of this kind become apparent when insight is sought into the mechanism of the behaviour. The explanation of this mechanism must be based on Richter's findings that self-regulation depends on intact taste perception. Below a critical blood glucose level the sense of taste for sweetness is obviously depressed. Only half of the subjects recognize a 5 per cent sucrose solution as sweet at all; almost three-quarters of the subjects, not recognizing the sweetness of saccharine, had a blood glucose below the critical level. The depression of taste perception affected sweet taste only; salt and bitter tastes (the latter tested in experiments with phenyl-thiourea, not reported in this paper) appeared undisturbed. As it is generally assumed that special receptors of the tongue react to only one taste (Moncrieff, 1944), such as sweet,

and not to sour, bitter or saline, a specific depression of one type of receptor seems possible. Further investigations on this point, using threshold solutions, seem desirable.

The second problem is raised by the finding of a critical level in the region of blood glucose 50, below which the sweet taste perception becomes depressed. We have previously suggested (Mayer-Gross and Walker, 1945) that the glucose level of 10–15 mg./100 ml. is the critical level for the specialized function of cells in the cerebral cortex. It may well be that the level of 50 mg./100 ml. represents the critical level for the function of the taste buds of the tongue concerned with the sense of sweet. The function is not entirely lost at this level, but a definite depression of activity occurs.

The third problem refers to the apparent refinement within the sweet taste range, enabling the subjects to distinguish between a 30 per cent solution of sucrose and an equally sweet solution of saccharine. Several explanations are possible: one is that, owing to the depression of the sweet taste component in saccharine, the minute admixture of bitter or metallic taste, for which perception is undisturbed, comes to the fore and facilitates discrimination—especially below the critical blood glucose level.

A second possible explanation makes use of the different physical properties of the two solutions: (*a*) sucrose is more viscous than the saccharine solution, and will therefore be washed off the less quickly; (*b*) as concentration of solids in the 30 per cent sucrose solution is great, their diffusion into the tissue of the tongue is likely to be greater than in the case of the saccharine solution, where the concentration of solids is small; (*c*) as the specific gravity of saccharine is lower than that of the sucrose solution, the latter will exert greater pressure on the highly sensitive touch receptors of the tongue, which may facilitate discrimination. These differences in physical properties are, of course, the same whatever the level of the blood sugar. If, however, we accept the theory of the depression of function of the taste buds for sweet below a blood glucose level of 50, it is possible that at the depressed level of activity subsidiary factors of this kind play a greater part in the perception as a whole, and favour the choice of the sugar solution.

The results furnish no explanation of the observations at variance with self-regulation, which we mentioned in the introductory remarks—namely, the infrequency of " hunger riot " in insulin treatment, and the occasional intractability and lack of insight of hypoglycaemic diabetics. To account for these observations one will have to resort to the general depression of the cerebral cortex manifesting itself in apathy, lack of spontaneity, or emotional impassiveness. Such symptoms naturally become imperceptible if the investigator elicits answers from the subject, as in our experiments.

The relatively rigid experimental procedure may also be responsible for the poor yield of information on personal predilections and individual differences. On less than ten occasions—some mentioned under saccharine—an abnormal reaction could be suspected to be of this nature or, perhaps, influenced by the underlying psychosis.

SUMMARY

The paper is based on 202 experiments carried out in 100 subjects with the object of testing self-regulation in different stages of hypoglycaemia. The subject

was offered a choice of five liquids: water, 5 per cent sucrose, 30 per cent sucrose, 0·5 per cent sodium chloride and saccharine; his preference, judgment of taste and other reactions were noted and his blood glucose determined.

The most important result was the preference for 30 per cent sucrose solution below the blood glucose level of 50 mg./100 ml. and the rejection of it above this critical level.

Other findings, such as the judgment of sweetness for 5 per cent sucrose and saccharine, were also found related to the blood glucose level. Saccharine, although of equal sweetness with 30 per cent sucrose, was rejected below the critical level.

Loss of water and chlorides during hypoglycaemia had no influence on the selection of liquids; nor had odour, as demonstrated in special experiments.

The results are discussed in the light of the principle of self-regulation; but an attempt has been made also to explain the mechanism of the reactions in terms of the physiology of taste perception.

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THE EFFECTS OF LEWISITE AND OF LEWISITE OXIDE ON LIVING CELLS *IN VITRO*.

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From the Strangeways Research Laboratory, Cambridge.

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THE experiments described below formed part of a research on the action of vesicants on living cells which was done at the Strangeways Research Laboratory for the Chemical Defence Research Department, Ministry of Supply, between 1939 and 1944.

Tissue cultures were grown *in vitro* under conditions which allowed the lewisite either to be applied to them superficially, or to be incorporated at known concentrations in the clots in which the cultures were growing. The effects on individual cells could be studied by continuous observation of the living tissue on a microscope kept at body temperature and equipped with a specially modified Mark VI service respirator, in which a 'blind' eye fitted over the

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microscope ocular. Alternatively, they could be investigated in cultures incubated in the usual way and examined or treated at pre-arranged intervals. By these means a fairly complete and detailed picture of the processes leading to the death of the cell could be obtained.

MATERIAL AND METHODS.

Cultures of the sclerotic from 11-day fowl embryos were grown in a mixture of equal parts of fowl plasma and embryo extract by the ordinary hanging-drop method. One and a quarter inch square coverslips mounted over 3 × 1½ in. hollow-ground slides were used.

RESULTS.

1. *Liquid Lewisite.*

After 48 hours' growth in normal medium the coverslips bearing the cultures were removed from the hollow-ground slides and each was placed in contact with a drop of lewisite on an ordinary flat slide, being supported on two strips of paraffin wax previously painted across the glass. Sixty-six cultures were treated.

Immediately the lewisite touched the plasma clot it emulsified and the clot appeared white and opaque. In the cells the fat globules began to swell, and soon afterwards the protoplasm suddenly coagulated so that the cell was "fixed" without much distortion of outline. The fat globules continued to swell rapidly, and about 15 mins. after the application of the lewisite the protoplasm of the cells near the surface of the clot had become completely infiltrated with droplets.

2. *Saturated Lewisite Vapour.*

Cultures were grown normally for 48 hours, each coverslip was then raised, a small drop of lewisite was placed in the hollow of the slide beneath but not in contact with the tissue, and the coverslip was resealed. Since the volume of the space under the coverslip did not exceed 3 c.c., it may be assumed that, at 37° C., equilibrium between vapour and liquid was attained almost instantaneously, so that the surface of the culture was in contact with saturated lewisite vapour. Twelve cultures were treated.

When observed on the warm stage, the cells were seen to be immediately coagulated, the form of nucleus and cytoplasm being even better preserved than after treatment with liquid lewisite. If a fairly large drop of lewisite was placed in the hollow of the slide the culture medium gradually developed a dense precipitate, as with the direct application of the liquid, the fat globules in the cells swelled, and eventually the whole cell became infiltrated with droplets.

3. *Slowly Diffusing (Unsaturated) Lewisite Vapour.*

Cultures were prepared in the usual way, except that a small glass tube was inserted in the wax seal of the coverslip ; one end of the tube opened into the culture vessel, the other was sealed with paraffin wax. After 48 hours' growth the culture was taken from the incubator, the seal removed from the tube, and a small drop of lewisite was placed near the outer end of the pipe, which was re-sealed. The culture was either returned to the incubator or watched on a warm stage. Vapour diffused slowly down the pipe into the culture vessel. Twelve cultures were used for this experiment.

When a culture was watched on the warm stage, the cell processes could soon be seen to retract, and at the same time small blebs appeared on the surface of the cytoplasm. Contraction of the cell continued, the fat globules fused into larger droplets, and small, round vacuoles appeared, especially near the margin of the cytoplasm. In some cells these peripheral vacuoles became very large, and eventually formed a balloon-like cyst on one side of the cell body.

About two hours after the first application of the vapour the cytoplasm had become very vacuolated, the nucleus was pycnotic, and both nucleus and cytoplasm had acquired a rather opaque, greenish appearance when viewed by transmitted light. The outline of the cells, though very contracted, was fairly regular. In the mitotic cells the chromosomes were clumped together and often fused into a single mass.

4. *Lewisite Oxide.*

(a) *The effect on cells of contact with a lethal solution.*

Cultures were grown for 48 hours and then placed in contact with a solution of lewisite oxide in Pannett and Compton's saline by the method described for the application of liquid lewisite. Two concentrations of lewisite oxide were used, viz. a saturated solution (19 cultures) and a concentration of 0·0056 mg. per c.c., or about twice the lethal dose (see next section) (20 cultures). As controls, contact preparations were made with Pannett and Compton's saline only (9 cultures).

The saturated solution had a rapid and drastic action on the cells. Five minutes after its application small blebs appeared on the surface of the cytoplasm, and usually broke off from the rest of the cell. After about fifteen minutes small, round vacuoles formed, especially near the nucleus; they enlarged and sometimes protruded from the surface like small balloons. The fat globules lost their compact arrangement on one side of the nucleus and became much more diffusely distributed, while the nucleus became shrunken and more opaque. One hour and a half after application the cells showed a curious phenomenon. The cell membrane remained intact, but in places the cytoplasm became very rarified and the cell gradually distended with fluid until it formed a small vesicle, the nucleus and more solid parts of the cytoplasm being compressed into a crescent in the vesicle wall. Eventually the cell membrane seemed to break down, leaving the crescentic remains of the protoplasm lying in a circular hole in the plasma clot.

The more dilute solution of lewisite oxide acted much more slowly than the saturated solution, and the general course of degeneration resembled that caused by slowly diffusing lewisite vapour.

(b) *Determination of the minimum lethal concentration.*

Cultures were grown in normal medium (equal parts of plasma and embryo extract) for 48 hours, and were then transferred to medium containing lewisite oxide. After a further 48 hours' incubation the cultures were fixed in acetic alcohol and stained with Ehrlich's haematoxylin.

The lewisite oxide was introduced into the culture medium in the following way: It was dissolved in Pannett and Compton's saline, and diluted to various concentrations; a constant volume of each solution was added to a constant volume of embryo extract. Each explant was grown in a clot composed of

0·015 c.c. embryo extract containing a known quantity of lewisite oxide + 0·015 c.c. fowl plasma.

Three experiments were made, in each of which several concentrations of lewisite oxide were tested and six cultures were grown in each concentration. In every experiment six control cultures were grown in plasma + embryo extract diluted with the usual volume of saline, but without lewisite oxide.

TABLE.—*Summary of Experiments on the Minimum Lethal Concentration of Lewisite Oxide.*

No. of experiment.	Concentration of lewisite oxide in culture medium (mg. per c.c.).	Result.
I	0·285	Cultures all dead.
	0·0285	,, ,,
	0·0029	,, ,,
	Controls	Growing well.
II	0·0028	All dead.
	0·00028	All showed fair growth.
	0·00003	Growing well.
	Controls	,, ,,
III	0·0028	All dead.
	0·00165	Few cells outwandering from each explant.
	0·00082	Poor, scanty outgrowth.
	0·00028	Fair growth.
	Controls	Growing well.

The results are summarized in the Table. In the first experiment the lowest concentration used was 0·0029 mg. per c.c., which killed all the cultures so that there was no outgrowth after 48 hours' incubation. In the second experiment 0·0028 mg. per c.c. was taken as the highest concentration and 0·00028 mg. per c.c. as the next. The former killed all the cultures, but the explants showed fairly good growth in the latter. Thus the lethal concentration lay somewhere between 0·0028 and 0·00028 mg. per c.c.

In the third experiment these two concentrations were selected as the highest and lowest respectively, and two intermediate concentrations, 0·00165 and 0·00082 mg. per c.c., were tested at the same time. As before, 0·0028 mg. of lewisite oxide per c.c. killed all the cultures, but in the next concentration, 0·00165 mg. per c.c., although most of the tissue was killed a few cells survived in each culture and wandered out into the clot. Still more cells survived in a concentration of 0·00082 mg. per c.c., and after 48 hours' incubation the explants were surrounded by a feeble, scanty zone of outgrowth.

These results showed, therefore, that for living cells *in vitro* the minimum lethal concentration of lewisite oxide was between 0·0028 and 0·00165 mg. per c.c.

DISCUSSION.

The results described above show that the first visible effect of liquid lewisite on living cells *in vitro* is a rapid swelling of the fat globules, owing to the high

fat solubility of the agent. This is quickly followed by a sudden precipitation or " fixation " of the protoplasm without much distortion of the cells, which is presumably due to the change in pH as the lewisite hydrolyses. A similar rapid precipitation of the protoplasm is caused by saturated lewisite vapour, but slowly diffusing vapour produces a softening and partial liquefaction of the cytoplasm.

The experiments with lewisite oxide emphasize the extreme toxicity of this substance, of which the minimum lethal concentration is as low as 0·0028 to 0·00165 mg. per c.c. Applied to normal cells lethal solutions of the oxide cause vacuolation and softening of the protoplasm, an effect which differs from that of liquid lewisite but resembles that of the vapour in low concentrations. It is possible, therefore, that the toxicity of the slowly diffusing vapour results, at least partly, from hydrolysis in the tissue fluids to form the water-soluble oxide.

SUMMARY AND CONCLUSIONS.

1. Liquid lewisite dissolves in the fat globules of cells with which it comes into contact, making the globules swell. It then causes a sudden precipitation of protoplasm, so that the cell becomes " fixed " with little distortion of its form. Saturated lewisite vapour behaves similarly.

2. Lewisite vapour diffusing *slowly* on to a cell causes contraction of both cytoplasm and nucleus, and vacuolation and softening of the cytoplasm.

3. Cultures placed in contact with a saturated solution of lewisite oxide undergo rapid and drastic changes. The nuclei become pycnotic, the cytoplasm vacuolated, and eventually the cells are reduced to vesicles, the nucleus and more solid parts of the cytoplasm being compressed in the vesicle wall. A dilute solution of lewisite oxide acts more slowly, and its effects resemble those caused by the slowly-diffusing lewisite vapour.

4. The *minimum* lethal concentration of lewisite oxide for cells growing *in vitro* is between 0·0028 and 0·00165 mg. per c.c. of culture medium—a very high toxicity.

One of us (C.B.A.) was in receipt of a personal grant from the Medical Research Council while this work was being done. We are indebted to the Director General of Scientific Research (Defence) of the Ministry of Supply for permission to publish the results.

THE THERAPEUTIC EFFECT OF BRITISH ANTI-LEWISITE (BAL) ON TISSUE CULTURES GROWN IN A MEDIUM CONTAINING LEWISITE OXIDE.

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ATTENTION has recently been directed (Peters, Stocken and Thomson, 1945) to the chemical properties and therapeutic use as an antidote for lewisite, of the dithiol compound 2 : 3–dimercaptopropanol, known as "British Anti-Lewisite" (BAL).

In the present paper a series of tissue culture experiments are described which were made on behalf of the Ministry of Supply in 1940–41 to find whether the toxic action of lewisite oxide on cells cultivated *in vitro* could be prevented or cured by suitable treatment with BAL.

MATERIAL AND METHODS.

Cultures of the sclerotic from 11-day chick embryos were grown by the usual hanging-drop method on 1¼-inch square cover-slips sealed with paraffin wax over 3 × 1½ inch hollow ground slides. All cultures were grown for 48 hours in normal medium composed of equal parts of cock plasma and 11-day chick embryo extract before being transferred to experimental media containing lewisite oxide, BAL, or both. To prepare the latter media, known quantities of the chemical agents were dissolved in Pannett and Compton's saline, and appropriate volumes of the solutions were then added to the normal mixture of plasma and embryo extract ; the same volumes of Pannett and Compton's saline only were added to the control cultures. Further details of the procedure are described in the accounts of individual experiments.

The cultures were fixed in acetic alcohol, stained with Ehrlich's haematoxylin, and mounted whole in Canada balsam.

RESULTS.

1. *The Toxicity of BAL to Cells Growing in Vitro.*

The cultures were grown in normal medium for 48 hours, and were then brought into contact with BAL. This was done by removing each culture coverslip from its hollow ground slide and remounting it on a flat slide in contact with a drop of a solution of BAL in saline. The coverslip was supported on two strips of paraffin wax previously painted on the slide, and its edges were sealed with wax. These contact preparations were incubated at 37° C., and examined under the microscope. Six cultures were used for each solution.

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TABLE I.—*Toxicity of BAL to Cells in vitro.*

Culture set.	Concentration of BAL.	Observation.
i	10·6 mg./c.c.	After 1 hour both cells and culture medium were coagulated and the cultures were dead.
ii	0·96 mg./c.c.	After 1 hour the cells were rather degenerate, but the effect was much less drastic than for (i). After 25 hours the cells were coagulated.
iii	0·09 mg./c.c.	After 1 hour the cells appeared fairly healthy. After 25 hours they were very degenerate, but not coagulated.

It was concluded that a concentration of 0·09 mg. per c.c. represents a just sub-lethal concentration of BAL for cells *in vitro* when applied for 24 hours.

2. *The Effect of BAL on Cultures Previously Treated with Lewisite Oxide.*

Experiment A.

In this experiment fifty-four cultures which had been grown for 48 hours in normal medium were transferred to one containing lewisite oxide in a concentration of 0·00125 mg. per c.c., and twelve controls were transferred to normal medium. After incubation at 37° C. for 24 hours, the cultures treated with lewisite oxide were very unhealthy and showed only a few small spikes of outgrowth, while the controls were growing normally. All but eighteen of the cultures were then transferred to contact preparations with concentrations of

TABLE II, A.—*Effect of BAL on Cultures Poisoned with Lewisite Oxide.*

Culture set.	Number of cultures.	Initial treatment.	Contact solution.	Final condition of cultures.
iv	6	Lewisite oxide	None	Only a few isolated cells emerged.
v	12	Lewisite oxide	Saline only	Either a few small spikes of outgrowth and scattered cells, or no growth.
vi	12	Lewisite oxide	BAL 0·025 mg./c.c.	Fairly good localized patches of outgrowth in two cultures; only small spikes or scattered cells in the remaining ten.
vii	12	Lewisite oxide	BAL 0·0025 mg./c.c.	Six cultures showed quite good outgrowth, sometimes with mitosis. The remainder showed a small fringe of cells.
viii	12	Lewisite oxide	BAL 0·00025 mg./c.c.	Three cultures had small, localized outgrowths. The remainder had either isolated cells or no growth.
ix	12	Normal medium	None	Broad zone of outgrowth.

BAL in saline for 1 hour, after which they were removed, and the excess fluid was sucked off with a pipette. These cultures were remounted over hollow-ground slides without change of culture medium, and incubated for a further 24 hours. The results are summarized in Table II, A.

These results showed that tissues cultivated in medium containing a nearly lethal concentration of lewisite oxide would partially recover if treated with BAL in suitable concentration (0·0025 mg./c.c.).

Experiment B.

In the technique described above, the contaminated cultures, after contact with BAL solution, were remounted without any attempt to remove either excess reagent or a possible product of its reaction with the lewisite. A second series of experiments was therefore made in which the cultures were incubated for 48 hours in a medium containing 0·00125 mg. of lewisite oxide per c.c., which, as before, nearly inhibited growth; they were then excised from the lewisite oxide-containing clots and placed in either a BAL solution or saline in watch-glasses. The watch-glasses, enclosed in moist chambers, were incubated at 37° C. for 20 minutes, after which the cultures were washed in several changes of Pannett and Compton's saline, before being transferred to fresh normal medium in hanging-drop preparations and incubated for a further 48 hours. The results are recorded in Table II, B.

TABLE II, B.—*Effect of BAL on Cultures Poisoned with Lewisite Oxide.*

Culture set.	Number of cultures.	Initial treatment.	Contact solution.	Final condition of cultures.
x	6	Lewisite oxide	Saline	3 cultures had a few straggling sprouts of outgrowth. The rest had no growth.
xi	6	Lewisite oxide	BAL 0·025 mg./c.c.	All growing. 4 had extensive zones of outgrowth with mitosis.
xii	6	Lewisite oxide	BAL 0·0025 mg./c.c.	4 showed fairly active outgrowth with mitosis, one had a few cells outwandering, and the sixth, no growth.
xiii	6	Lewisite oxide	BAL 0·00025 mg./c.c.	2 showed fairly good but localized outgrowth, 3 had a few spikes, and 1 no growth.
xiv	6	Normal medium	None	Grown in normal medium throughout; all had a broad zone of outgrowth.

It is seen from Table II, B, that the results of Experiment B confirmed those of Experiment A in showing that BAL exercised a curative action on the poisoned cells. There was a difference, however, between the most effective concentrations of BAL in the two experiments, the optimum concentration in A being 0·0025 mg./c.c. (set vii) as compared with 0·025 mg./c.c. in B (set xi). This difference may be attributed to the fact that in Experiment A, set vi was exposed to the somewhat toxic action of 0·025 mg./c.c. BAL for 1 hour, after

which the tissue was neither washed nor transferred to fresh culture medium, whereas set xi was in contact with this concentration for 20 minutes only, and was then washed and transplanted to fresh medium. The most dilute solution (0·00025 mg./c.c. BAL) was the least effective in both experiments, and was probably too dilute to destroy the lewisite oxide.

3. *The Time Factor in the Therapeutic Treatment with* BAL *of Lewisite Oxide-poisoned Cultures.*

In view of the results described in section 2, experiments were undertaken to find whether BAL in a concentration which was effective in destroying lewisite oxide in contaminated tissue might itself have a toxic action if left in contact with the cells for too long, so that a short treatment with the more concentrated BAL solutions might have a curative effect which would be masked after a long treatment.

The technique was essentially the same as in Experiment B (section 2). For the first group of experiments in the present series, cultures were grown for two days in normal medium after which some were transferred for a further 48 hours to medium containing 0·00125 mg./c.c. lewisite oxide and others to normal medium.

TABLE III, A.—*Effect of Varying Time of Contact on the Therapeutic Effect of BAL on Cultures Poisoned with Lewisite Oxide.*

Culture set.	Number of cultures.	Initial treatment.	Immersion fluid.	Duration of immersion.	State of cultures after final growth for 48 hours in normal medium.
xv	12	Lewisite oxide	Saline only	2 hours	There was no significant difference between sets (xvii, xix) receiving longer contact with BAL and those having shorter contacts (xviii, xx); all cultures treated with BAL grew far better than those (xv, xvi) washed in saline only.
xvi	12	Lewisite oxide	Saline only	20 mins.	
xvii	12	Lewisite oxide	BAL 0·025 mg./c.c.	2 hours	
xviii	12	Lewisite oxide	BAL 0·025 mg./c.c.	20 mins.	
xix	6	Lewisite oxide	BAL 0·25 mg./c.c.	20 ,,	
xx	6	Lewisite oxide	BAL 0·25 mg./c.c.	5 ,,	
xxi	6	Lewisite oxide	BAL 2·5 mg./c.c.	20 ,,	These sets also grew better than sets xv and xvi. Set xxii was in slightly better condition than set xxi, but not significantly so.
xxii	6	Lewisite oxide	BAL 2·5 mg./c.c.	5 ,,	
xxiii	12	Normal medium	Saline only	2 hours	

They were then excised from the clot, immersed for various periods in either a BAL solution or Pannett and Compton's saline and transferred to normal culture medium; after two days' incubation they were fixed and stained. The results are summarized in Table III, A.

In the second group of experiments (section 3), all the cultures were immersed for 5 mins. at 37° C. in a solution containing 2·5 mg. of BAL per c.c. They were then transferred either to normal medium or to a medium containing 2·5 mg. of BAL per c.c. of clot and grown for a further 48 hours.

TABLE III, B.—*Comparison of Therapeutic Effects of Long and Short Contacts with BAL*

Culture set.	Number of cultures.	Initial treatment.	Immersion fluid (5 mins.).	Final culture medium.	State of cultures after 48 hours in final medium.
xxiv	10	Normal medium	Saline	Normal	All growing well.
xxv	10	Lewisite oxide	Saline	Normal	3 had a few scattered cells; 7 no growth.
xxvi	10	Normal medium	BAL 2·5 mg./c.c.	Normal	Growth almost as good as set xxiv.
xxvii	12	Lewisite oxide	BAL 2·5 mg./c.c.	Normal	All growing, with mitosis. Some almost as good as set xxiv.
xxviii	10	Normal medium	BAL 2·5 mg./c.c.	Normal + BAL 2·5 mg./c.c.	All dead after 24 hrs. Cells and medium coagulated.
xxix	12	Lewisite oxide	BAL 2·5 mg./c.c.	Normal + BAL 2·5 mg./c.c.	All dead and coagulated after 24 hours.

It is clear from these results that 5 minutes' application of BAL at the high concentration of 2·5 mg./c.c. had a very beneficial effect on the cultures injured by lewisite oxide (compare set xxvii with set xxv), but 24 hours' contact with the same concentration of BAL was completely lethal to all cultures (sets xxviii and xxix). The short application of BAL at this concentration to normal tissue (set xxvi) had little deleterious effect.

4. *The Protective Action of BAL on Tissue Cultures Grown in a Medium Containing Lewisite Oxide.*

An experiment was made to find whether tissue explanted into culture medium containing lewisite oxide could be protected from the effect of the toxin by the simultaneous addition to the medium of a sub-toxic concentration of BAL. Cultures were grown in a normal medium for 2 days and then transferred for a further 48 hours to media containing lewisite oxide (0·00125 mg. per c.c.), either alone or with various proportions of BAL. Six cultures were used in each set. The results were as follows (Table IV):

TABLE IV.—*Protective Action of BAL Against Poisoning of Cultures by Lewisite Oxide.*

Culture set.	Concentration of Lewisite oxide in final medium.	Concentration of BAL in final medium.	State of cultures after 48 hours in final medium.
xxx	*Nil*	*Nil*	Normal medium throughout; active growth in all cultures.
xxxi	0·00125 mg./c.c.	*Nil*	No growth.
xxxii	0·00125 mg./c.c.	0·0125 mg./c.c.	Growing well; outgrowths only slightly less than in set xxx. Many abnormal mitoses (arrested metaphase).
xxxiii	0·00125 mg./c.c.	0·00125 mg./c.c.	No growth.
xxxiv	*Nil*	0·0125 mg./c.c.	Almost as good as set xxx.
xxxv	*Nil*	0·00125 mg./c.c.	Almost as good as set xxx.

Thus, a concentration of 0·0125 mg./c.c. of BAL gave almost complete protection against a concentration of lewisite oxide ten times less, but the lower concentration of BAL was ineffective.

SUMMARY.

1. The lethal concentration of BAL applied for 24 hours to cells growing *in vitro* is of the order of 0·09 mg./c.c.

2. Tissue cultures severely poisoned with a concentration of lewisite oxide which is just sub-lethal (0·00125 mg./c.c. in the clot) largely recover when treated with a concentration of BAL of the order of ten times that of the lewisite oxide.

3. Very short application of a heavy concentration of BAL (2000 times that of the lewisite oxide), itself lethal on longer contact, leads to good recovery of cultures poisoned as in 2.

4. Under the experimental conditions described, a 10 : 1 excess of BAL in the culture medium almost completely protects cells from the action of a just sub-lethal concentration of lewisite oxide.

We acknowledge our indebtedness to Professor R. A. Peters, F.R.S., for his interest in, and friendly criticism of this work. Our thanks are due to the Director-General of Scientific Research (Defence), Ministry of Supply, for permission to publish the results.

One of us (C.B.A.) was in receipt of a personal grant from the Medical Research Council while this work was being done.

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THE STATE OF VITAMIN A IN HUMAN SERUM.

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IT has been stated (Chevallier and Choron, 1938; Clausen, Baum, McCoord, Rydeen and Breeze, 1942; Morton) that in human serum the vitamin A is present as the free alcohol. On the other hand the vitamin is stored in the liver mainly as esters (Clausen and McCoord, to be published). When ingested the vitamin is absorbed from the gut in the form of the free alcohol (Gray, Margarlidge and Cawley, 1940), but it seems that it is re-estified during or soon after the absorption (Drummond, Bell and Palmer, 1935). In the work reported here the distribution of vitamin A between the free and esterified form was determined in normal sera and in sera obtained under conditions in which the vitamin A level was temporarily raised.

METHODS.

All colour measurements were carried out in a photo-electric photometer of the Evelyn type (Evelyn, 1936) which has been modified (Hoch, 1944). The optical containers were selected cylindrical centrifuge tubes of about 8 mm. internal diameter, and they were marked at 1 ml. The results are expressed in extinctions, $\log_{10} \frac{Io}{I}$, Io and I being the galvanometer readings (corrected for non-linearity of the galvanometer) given by solvent and solution respectively.

The reagent for vitamin A which was found satisfactory and most convenient to prepare was a solution of $SbCl_3$ in $CHCl_3$† that had been boiled (Dann and Evelyn, 1938) in the presence of an excess of the salt and left to stand at room temperature (18–22° C.). Some $SbCl_3$ crystallized out, and on keeping, two layers formed, the upper of which was used. Reagents prepared from two different batches (*a*) and (*b*) supplied by one firm (Hopkin & Williams, Ltd.), and from one batch (*c*) supplied by another firm (B.D.H. Ltd.) gave the same extinctions with solutions of a liver oil concentrate, but widely differing results with pure β-carotene (relation of extinctions (*a*) : (*b*) : (*c*) = 1 : 1·26 : 1·8). Since differences in the solubilities of $SbCl_3$ might cause variation in the "blue" values of carotenoids, the contents in $SbCl_3$ of solutions obtained at various temperatures were determined by titration with iodine (Gstirner, 1940). The results given in Table I show that the solubilities of the products from the two firms are very similar, and that under the conditions of preparation and use of the solutions no appreciable oversaturation could have occurred. They also indicate that at temperatures higher than 18° C. the colour given with vitamin A

* Part of this work was done during tenure of a scholarship from the Freedom Research Fund of the London Hospital.

† Chloroform *pro anaesthesia*, containing 1·5 per cent ethanol.

TABLE I.—*Comparison of Solubilities of Samples of $SbCl_3$ of Different Provenance.*

Batch.	Time of standing before sampling for analysis.	Temperature.	% $SbCl_3$.
(*b*)	2 days	23°	32
	Same sample cooled for 10 mins.	18°	30
	Same sample cooled during 2·5 hours from	18 to 16°	26
(*c*)	1 day	22°	35
	16 hours	18°	27
	1 hour	15·5°	26

(0·8 ml. reagent to 0·3 ml. extract) may be estimated to be at least 99 per cent of the maximum colour according to the relationship between colour and concentration of $SbCl_3$ given by Dann and Evelyn (1938). The reaction given by β-carotene exhibited different colours during the first two seconds after mixing: purple-brown in the case of batches (*a*) and (*b*), and blue in that of batch (*c*). Batches (*a*) and (*b*) gave maximum readings before 10 secs., batch (*c*) after 40 secs. In the experiments described here only one single batch of $SbCl_3$ (batch (*b*)) was used, and no variation in the "blue" values given by β-carotene was found over a period of 16 months, irrespective of whether the reagents used were made up fresh or whether they had been kept (Table II).

TABLE II.—*"Blue" Values of some Carotenoids Occurring in Human Serum.*

Carotenoid.	(1) $\log \frac{Io}{I}$ violet.	(2) $\log \frac{Io}{I}$ orange.	Ratio $\frac{(2)}{(1)}$.	Date.	Reagent.
β-carotene (cryst.)	0·812	0·096	0·118	2.3.44	Freshly made up
	0·805	0·098	0·122		
	0·843	0·096	0·114		On keeping 8 days
	0·812	0·099	0·122		
	0·444	0·051	0·115	17.3.44	Batch (*a*)
	0·976	0·115	0·118	12.4.44	
Lycopene (serum)	0·323	0·033	0·102	10.3.44	
Lutein 1 (serum)	0·551	0·077	0·140	2.3.44	
Lutein 2 (serum)	0·305	0·049	0·161	2.3.44	On keeping
	0·488	0·079	0·162		
	0·707	0·119	0·168	17.3.44	
	0·560	0·085	0·152		
β-carotene (cryst.)	1·039	0·141	0·136	12.4.44	On keeping, batch (*b*).
	0·692×2	0·189	0·136	25.7.44	
	0·706×2	0·188	0·133		
	0·551×2	0·150	0·136	15.8.44	
	0·359	0·048	0·134	2.11.45	
	0·726	0·100	0·138	4.11.45	
	0·540	0·075	0·139		
	0·729	0·100	0·137		
β-carotene (serum)	0·540	0·073	0·135	16.5.46	
Lycopene (tomatoes)	0·749×2	0·174	0·116	15.8.44	
Lutein 2 (serum)	0·806	0·108	0·134	2.11.45	
	0·826	0·112	0·136		
	0·538×2	0·156	0·145	29.4.46	
Lutein (marigold)	0·829×2	0·280	0·169	25.7.44	
	0·667×2	0·232	0·174		

Determination of total vitamin A.

The extraction was carried out by a method similar to that of Skurnik and Suhonen (1939). To 1 ml. of serum (or plasma) contained in a narrow-necked conical centrifuge tube were added in succession with immediate mixing 0·15 ml. of 29 per cent NaOH and 0·5 ml. ethanol. The air was replaced by nitrogen, the tube stoppered tightly and the mixture kept in a boiling water bath for 5 mins. It was left to cool, centrifuged shortly, about 5 drops of light petroleum were added and mixed with the contents of the tube. Following this addition no or very little precipitate was seen. A further 3 ml. petrol and 0·64 ml. ethanol were added, without mixing. The tube was stoppered immediately and the contents were well shaken. Two further extractions with about 2 ml. each of light petroleum removed vitamin A and carotenoids quantitatively. Centrifuging for not more than 20 secs. was needed to separate the layers. The pooled extracts were shaken three times with H_2O, once with Fe-free 10 per cent HCl and again three times with H_2O.* The bottom layer was removed after each washing with a capillary pipette. The extract was transferred to a dry centrifuge tube, anhydrous Na_2SO_4 was added and the extract was evaporated to dryness in a stream of nitrogen, the tube being kept immersed in a water bath of 50–55° C. The extract was transferred to a photometer tube with enough " n-hexane " to make 1 ml. solution, and the carotenoids were estimated by their extinction in the violet (Ilford Spectrum No. 601 and 1·6 mm. thickness of Chance's Calorex glass) before the $SbCl_3$ reaction was carried out. The hexane was then evaporated in a stream of nitrogen; 0·1 ml. $CHCl_3$ was added and evaporated in nitrogen three times in order to remove all the hexane; finally 0·3 ml. $CHCl_3$ and 0·017 ml. acetic anhydride were added and the reaction was carried out with 0·8 ml. of the $SbCl_3$ reagent. The contents of the tube were mixed immediately, the tube placed into the photometer (2 filters Ilford Spectrum No. 607 and 1·6 mm. thickness of Chance's Calorex glass) and the galvanometer read 10 secs. after the $SbCl_3$ had been added. The procedure was carried out speedily in order to allow the tube to be in correct position for about 5 sec. before the reading was taken. The mixing was effected by two or three taps to the tube so as to avoid bubbles remaining in the region of the optical path. Further readings were obtained at intervals of 10 sec. for 1 min. in order to check the first reading by extrapolation. The values obtained after 10 sec. were slightly lower than the maximum values which occurred earlier. The volume of the mixture was determined by measuring the distance of the meniscus from the 1 ml. mark on the photometer tube, to an accuracy of 0·2 mm., i.e. about 1 per cent, and the value of the extinction was corrected to the standard volume of 1·1 ml. This technique obviates the difficulty of delivering quickly accurate volumes of the reagent, and the errors introduced by the varying volumes of the extracted materials. A calibration curve was prepared with solutions of a liver oil concentrate containing 170,000 i.u./g. The apparent rate of fading was 0·6 per cent/sec. The effects of light intensity on the stability of the $SbCl_3$ reaction have been discussed by Caldwell and Parrish (1945). A value corresponding to the extinction in the orange due to carotenoids was calculated from the extinction in the violet, and it was subtracted from the total extinction obtained in the $SbCl_3$ reaction. The uncertainty involved in this procedure of correcting for a colour reaction due to carotenoids arises from

* The reason for the use of HCl was pointed out earlier (Hoch, 1943).

the possible presence of (1) unknown carotenoids or cis-trans isomers of those present, (2) colourless oxidation products of carotenoids other than vitamin A, which give a colour with $SbCl_3$, or (3) substances in serum extracts which might alter the rate of colour development or fading in the reaction of carotenoids with $SbCl_3$.

The reproducibility of measurements by the $SbCl_3$ reaction as described above was tested on a solution of vitamin A. Ten determinations gave a mean extinction of 0·1444 and a range of 0·0046. The standard deviation worked out to $= 0·0015$ and the coefficient of variation to 1 per cent. Only one of the ten values (0·1476) fell beyond the range of $\pm$ 2 per cent.

Chromatographic separation of vitamin A alcohol and its esters.

Chromatography on Al_2O_3 has been used by Reed, Wise and Frimott (1944) in the analysis of liver oils, although they consider this method not feasible for the analysis of serum. The adsorbent used in the present experiments was prepared from a sample Al_2O_3, according to Brockmann (manufactured by Savory & Moore, London, W. 1), the activity of which had been reduced by exposure to moisture of the air, and another sample which had been activated by heating until no more moisture was given off. These two were mixed in such proportion that chromatograms of serum extracts showed no or little separation of neo-lycopene A from β-carotene, but a clear separation from lycopene. The activity of the Al_2O_3 was also controlled by observing the behaviour on it of a chromatographically pure fraction prepared from commercial Sudan III (Brockmann and Schodder, 1941). The method of extraction without saponification and the appearance on the column of the carotenoids in the presence of fat has been described in an earlier paper (Hoch, 1944). The Al_2O_3 used in the present experiments, however, was considerably less active. The serum extract was dissolved in as little n-hexane as possible and transferred to the adsorption column. For elution n-hexane and acetone in n-hexane in concentrations of 0·25 to 25 per cent were used. Four or five fractions were collected, and each fraction was treated with NaOH, etc., as described above for total vitamin A. The ratios of extinctions in the violet and in the orange of individual carotenoids which were used in correcting for the colour given by them in the $SbCl_3$ reaction were determined on (*a*) recrystallized, chromatographically homogeneous β-carotene, (*b*) β-carotene isolated from human serum, (*c*) lycopene isolated from human serum, and (*d*) the two major carotenoids, which are absorbed at the top of the adsorption column (probably luteins) isolated from human serum. For comparison the values for lycopene isolated from tomatoes and a lutein isolated from marigold are included (Table II). It must be emphasized that the values of extinctions given, particularly those of the carotenoids in the violet, are sensitive to small alterations in the transmission characteristics of the light filters used and in the emission spectrum of the light source, and to differences in the spectral sensitivity distribution of photocells (or, possibly, of different places on the surface of one photocell) so that they are applicable only under well defined experimental conditions. The factors used for calculating the extinction in the orange (in 1·1 ml. solution) from that in the violet (in 1 ml. solution) were: were for fractions containing β carotene, 0·136; lycopene, 0·10; lutein, 0·138; lycopene+lycopene isomers, 0·10; β-carotene + lycopene isomers

+ lycopene, 0·125*; fractions between lycopene and lutein (0·137)†; total carotenoids, 0·137. The good agreement of the conversion factor for β-carotene with the value of 0·14 obtained by Dann and Evelyn (1938) must be considered fortuitous, in view of the varying properties of commercial $SbCl_3$. The results of an analysis of a saponified extract of serum are shown in Table III. All the blue

TABLE III.—*Distribution in the Fractions of a Completely Saponified Serum Extract of Substances other than Carotenoids which give a Colour Reaction with $SbCl_3$.*

Subject: N. C—, ♂, 27.8.45, 3 ml. serum used.

Fraction.	(1) $\log \frac{Io}{I}$ violet.	(2) $\log \frac{Io}{I}$ orange.	(3) Calculated correction for carotenoids.	"Vitamin A" (2)–(3).
β-carotene neo-lycopene A	0·093	0·015	0·012	0·003
Lycopene	0·033	0·007	0·003	0·004
Fraction III*a*	0·016	0·005	0·002	0·003
Fraction III*b*	0·047	0·402	0·006	0·396
Lutein	0·053	0·016	0·007	0·009
Total	0·242	0·445	0·030	0·415
1/3 of above total	0·081	0·148	0·010	0·138
Total on separate sample by routine method	0·100	0·170	0·014	0·156
Recovery	—	—	—	88·5%

colour (extinction in the orange) given with $SbCl_3$ by the first fractions including lycopene could be accounted for within the error of measurement by the carotenoids present. In non-saponified extracts the distribution is different (Table IV). It is concluded that in the non-saponified extracts the excess of extinction in the orange of the first fractions over that calculated to be given by the carotenoids is mainly due to esterified vitamin A. The separation on the column of vitamin A alcohol and its esters is quantitative, and in extracts of normal sera there is little vitamin A in the lycopene fraction and none in the colourless fraction immediately above it. The separation is evident if the chromatogram is viewed in ultraviolet light. The vitamin A alcohol is eluted with the carotenoid fraction (III*b*) between lycopene and lutein (With, 1941). A small amount of material giving a purple colour with $SbCl_3$ is found with the top fractions on the column.

The routine procedure adopted for the separation of the vitamin A esters from vitamin A alcohol was: 2 to 4 ml. of serum (or plasma) were extracted according to the method described for carotenoids. The extract, dissolved in n-hexane, was chromatographed on columns of Al_2O_3, 20 mm. long and 3 mm. in diameter. If the lower fraction was separated so as to include the lycopene it contained all the vitamin A esters and none of the vitamin A alcohol. The upper part of the column was eluted with a small amount of ethanol. In order to show

* In the average 30 per cent of the extinction in the violet of the first fraction was found to be due to lycopene and neo-lycopene A.

† This factor is provisional. The separation of this carotenoid from vitamin A has not yet been possible and work is still in progress. The purest fraction so far obtained gave a factor of 0·30.

TABLE IV.—*Distribution in the Fractions of Non-Saponified Serum Extracts of Substances other than Carotenoids which give a Colour Reaction with $SbCl_3$.*

Subject.	Fraction.	(1) $\log \frac{Io}{I}$ violet.	(2) $\log \frac{Io}{I}$ orange.	(3) Calculated correction for carotenoids.	"Vitamin A" (2)—(3).
N. C—, ♂, 27.8.45 Same serum as in Table III; 3 ml. serum used	β-carotene + neo-lycopene A	0·120	0·072	0·016	0·056
	Lycopene + fr. III*a*	0·033	0·011	0·003	0·008
	Fr. III*b*	0·051	0·356	0·007	0·349
	Lutein	0·052	0·016	0·007	0·009
	Total	0·256	0·455	0·033	0·422
	1/3 of above total	0·085	0·152	0·011	0·141
	Total on separate sample by routine method	0·100	0·170	0·014	0·156
	Recovery	—	—	—	90·5%
N. K—, ♀ 4 ml. serum used	β-carotene + neo-lycopene A	0·164	0·051	0·022	0·029
	Lycopene	0·059	0·025	0·006	0·019
	Fr. III	0·087	0·268	0·012	0·256
	Lutein	0·087	0·022	0·012	0·010
	Total	0·397	0·366	0·052	0·314
	1/4 of above total	0·099	0·092	0·013	0·079
	Total on separate sample by routine method	0·119	0·099	0·016	0·083
	Recovery	—	—	—	95%
P. C—, ♀ 3 ml. serum used	β-carotene	0·169	0·054	0·023	0·031
	Neo-lycopene A + lycopene	0·040	0·010	0·004	0·006
	Fr. III*a*	0·014	0·004	0·002	0·002
	Fr. III*b*	0·107	0·270	0·015	0·255
	Lutein	0·091	0·023	0·013	0·010
	Total	0·420	0·360	0·057	0·304
	1/3 of above total	0·140	0·120	0·019	0·101
	Total on separate sample by routine method	0·164	0·120	0·022	0·098
	Recovery	—	—	—	103%
D. F—, ♀, post delivery; 3 ml. serum used	β-carotene	0·105	0·047	0·014	0·033
	Neo-lycopene A + lycopene	0·061	0·030	0·006	0·024
	Fr. III*a*	0·025	0·012	0·003	0·009
	Fr. III*b*	0·105	0·292	0·014	0·278
	Lutein	0·061	0·015	0·008	0·007
	Total	0·357	0·396	0·045	0·351
	1/3 of above total	0·119	0·132	0·015	0·117
	Total on separate sample by routine method	0·148	0·135	0·020	0·115
	Recovery	—	—	—	102%
N. B—, ♀, coronary thrombosis, diabetes mellitus; 3 ml. serum used	β-carotene	0·238	0·094	0·032	0·062
	Neo-lycopene A + lycopene	0·230	0·037	0·023	0·014
	Fr. III*b*	0·120	0·358	0·016	0·342
	Lutein	0·211	0·065	0·029	0·036
	Total	0·799	0·554	0·100	0·454
	1/3 of above total	0·266	0·185	0·033	0·151
	Total on separate sample by routine method	0·323	0·190	0·044	0·146
	Recovery	—	—	—	104%

that no vitamin A alcohol was carried down in the fat contained in the first fractions, the following experiment was carried out: 4 ml. of serum were extracted. The bottom fraction was separated by chromatography irradiated with ultra-violet light to destroy most of the vitamin A, and re-chromatographed. This fraction, containing fat, β-carotene, neo-lycopene A, lycopene and possibly still some vitamin A esters was then divided into two equal parts. One part (*a*) was mixed with an aliquot of the original top fraction, corresponding to 1·5 ml. serum, which contained vitamin A present as alcohol. This mixture and the second part (*b*) of the irradiated fraction were chromatographed separately under the conditions described for the routine procedure and the first fractions were collected. The $SbCl_3$ reactions given by these two fractions yielded nearly identical values: 0·025 and 0·027 for parts (*a*) and (*b*) respectively.

The recoveries of vitamin A after chromatography ranged from 80 to 104 per cent., 28 out of 36 from 84 to 100 per cent of the values obtained by the direct method for total vitamin A; the recoveries of carotenoids after chromatography were in the mean 96 per cent. Two figures are given for the proportion of vitamin A esters: (*a*) assuming that the esters are not destroyed during chromatography, and (*b*) assuming that they and the vitamin A alcohol are destroyed in proportion to their respective concentrations in the original mixture. The headings "Vitamin A alcohol" and "Vitamin A esters" include all non-carotenoid material which gave an extinction with $SbCl_3$ in the orange.

Conversion factors.

An extinction of 0·10 in the violet corresponded to 0·62 μg. β-carotene/ml. An extinction of 0·10 in the orange was equivalent to 1·05 i.u. vitamin A/1·1 ml. of reaction mixture. The deviations from proportionality of extinction to concentration were less than 1 and 2 per cent within the range used in the analyses of sera for β-carotene and vitamin A respectively. The one high figure for vitamin A esters in Table VIII had been corrected for the deviation from proportionality.

Sampling of the blood.

Venous blood was obtained with minimal or no stasis and serum was used in all cases except one, in which oxalated plasma was used.

The estimation of serum proteins.

The estimation of serum proteins was carried out as a check on possible dilution or concentration of the blood sample due to stasis and other causes. The specific gravity method of Linderstrøm-Lang (Hoch and Marrack, 1945) was used. The presence of alcohol in the serum in the alcohol experiment (Table VII) can have interfered only to a negligible extent, since its concentration in the serum most probably did not exceed 0·2 per cent.

RESULTS.

Normal sera.—Table V shows that in normal sera about 10–20 per cent of the total vitamin A is present in the form of esters. There is no indication of a sex difference, and the range is remarkably small. In the one case (H. H.) studied over a long period the level of the esters was constant.

TABLE V.—*The Proportion of Vitamin A in the Form of Esters in Normal Human Serum.*

Subject.	Date.	Total vitamin A. log. $\frac{Io}{I}$/ml. serum. (1)	Vitamin A alcohol. log. $\frac{Io}{I}$. (2)	Vitamin A esters. log. $\frac{Io}{I}$. Found. (3)	Vitamin A esters. log. $\frac{Io}{I}$. per ml. serum.	Amount of serum used for extraction (ml.). (4)	Proportion of esters. % of total. (a) Before chr.* $\frac{(3)}{(1)\,.\,(4)} \times 100.$	Proportion of esters. % of total. (b) After chr. $\frac{(3)}{(2)+(3)} \times 100.$	Recovery %. $\frac{(2)+(3)}{(1)\,.\,(4)} \times 100.$	Total carotenoids. log. $\frac{Io}{I}$/ml. serum before chr.	β-carotene + neo-lycopene + lycopene. % of total carotenoids before chr.	Recovery after chr. %.
N. O—, ♀	11.4.46	0·131	0·422	0·058	0·015	4·00	11·1	12·1	92	0·173	41	99
N. K—, ♀	3.9.45	0·083	0·266	0·048	0·012	4·00	14·5	15·3	95	0·129	47	93
P. C—, ♀	21.8.45	0·098	0·267	0·037	0·012	3·00	12·6	12·2	103	0·177	43	95
M. G—, ♂	11.4.46	0·111	0·363	0·044	0·011	4·00	9·9	10·8	92	0·146	56	95
J. S—, ♂	11.4.46	0·160	0·405	0·051	0·017	3·00	10·6	11·2	95	0·133	48	97
M. M—, ♂	2.4.46	0·145	0·406	0·069	0·017	4·00	11·9	14·5	82	0·158	44	96
N. N—, ♂	25.3.46	0·130	0·407	0·084	0·021	4·00	16·1	17·1	94	0·144	34	90
N. C—, ♂	27.8.45	0·156	0·358	0·074	0·025	3·00	15·8	17·1	92	0·111	48	93
H. H—, ♂	7.8.45	0·155	0·666	0·083	0·017	5·00	10·7	11·2	97	0·204	51	92
,,	18.3.46	0.147	0.487	0·066	0·017	4·00	11·2	11·9	94	0·130	47	96
,,	5.4.46	0·145	0·463	0·074	0·019	4·00	12·7	13·8	93	0·129	49	92
,,	11.6.46	0·156	0·400	0·047	0·016	3·00	10·0	10·5	95	0·162	45	100
,,	29.7.46	0·154	0·401	0·059	0·020	3·00	12·8	12·8	100	0·210	55	97
E. M. J—, ♂	11.7.46	0·130	0·335	0·046	0·015	3·00	11·8	12·1	98	0·215	47	99

* chr. = chromatography.

TABLE VI.—*Serum Levels of Vitamin A*

Subject.	Time of sampling before and after parturition.	Serum proteins. g/100 ml.	Total vitamin A. log. $\frac{Io}{I}$/ml. serum.	Vitamin A alcohol. log. $\frac{Io}{I}$.		Vitamin A esters, log. $\frac{Io}{I}$.	
				Found.	per ml. serum.	Found.	per ml. serum.
			(1)	(2)		(3)	
A. F—	1 hr. before	6·55	0·055	0·104	0·041	0·024	0·0095
	6 days after	6·79	0·073	0·144	0·058	0·024	0·0095
V. N—	2 days before	6·97	0·041	0·111	0·028	0·044	0·0110
	6 days after	6·97	0·110	0·378	0·095	0·041	0·0103
R. B—	5 hrs. after	6·20	0·138	0·159	0·120	0·021	0·016
	5 days „	7·29	0·137	0·229	0·115	0·029	0·015
	11 „ „	6·90	0·135	—	—	—	—

TABLE VII.—*Effects of Various Doses of Ethanol on*

Subject (date).	Dose of ethanol. ml.	Time before and after dose given.	Serum proteins. g/100 ml.	Total vitamin A. log. $\frac{Io}{I}$/ml. serum.	Vitamin A alcohol. log. $\frac{Io}{I}$.	
					Found.	per ml. serum.
				(1)	(2)	
H. H— (25.3.46)	20	30 mins. after	Plasma used	0·178	0·371	0·124
		4 hours after		0·158	0·335	0·118
H. H— (5.4.46)	40	2 mins. before	7·20	0·145	0·463	0·116
		60 mins. after	7·36	0·159	0·498	0·125
		155 „	7·25	0·156	0·495	0·124
M. M— (2.4.46)	60	2 mins. before	6·80	0·145	0·406	0·102
		30 mins. after	6·77	0·163	0·489	0·122
		150 mins. after	6·89	0·171	0·593	0·148

TABLE VIII.—*Effects of the Oral Administration of Vitamin A*

1. One single dose of halibut liver oil, containing about 100,000 i.u.
2. One single dose of about 5000 i.u. vitamin A alcohol.

Subject.	Date.	Time before and after dose given.	Serum proteins. g/100 ml.	Total vitamin A. log. $\frac{Io}{I}$/ml. serum.	Vitamin A alcohol. log. $\frac{Io}{o}$.	
					Found.	per ml. serum.
				(1)	(2)	
1. H. H—	25.6.45	4 hours after	7·29	0·417	0·393	0·140
		7¼ „	7·40	0·550	0·466	0·130
	7.8.45	43 days after	7·31	0·155	0·666	0·133
2. H. H—	11.6.46	3½ hours before	7·35	0·156	0·400	0·133
		4 hours after	7·32	0·189	0·404	0·135
		6¼ „	7·35	0·184	—	—
3. E. M. J—	11.7.46	¼ hour before	7·25	0·130	0·335	0·112
		4¼ hours after	7·35	0·261	0·347	0·116
		6¼ „	7·44	0·193	0·391	0·130
4. H. H—,	29.7.46	¼ hour before	7·47	0·154	0·401	0·134
		4 hours after	7·47	0·329	0·423	0·141
		6 „	7·33	0·320	0·435	0·145
		16 „	7·06	0·184	0·431	0·144

Alcohol and Esters Before and After Parturition.

Amount of serum used for extraction. ml. (4)	Proportion of esters. % of total. (a) Before chr. $\frac{(3)}{(1)\,.\,(4)} \times 100.$	(b) After chr. $\frac{(3)}{(2)+(3)} \times 100.$	Recovery %. $\frac{(2)+(3)}{(1)\,.\,(4)} \times 100.$	Total carotenoids. log. $\frac{Io}{I}$/ml. serum before chr.	β-carotene + neo-lycopane + lycopene. % of total carotenoids before chr.	Recovery after chr. %.
2·52	17·3	18·7	92	0·176	28	100
2·50	13·1	14·3	92	0·113	31	92
4·00	26·8	28·4	94	0·077	34	92
4·00	9·3	9·8	95	0·077	35	99
1·33	11·4	11·7	98	0·371	43	98
2·00	10·5	11·2	94	0·376	44	100
—	—	—	—	—	—	—

the Levels of Vitamin A Alcohol and Esters.

Vitamin A esters. log. $\frac{Io}{I}$. Found. (3)	per ml. serum.	Amount of serum used for extraction. ml. (4)	Proportion of esters. % of total. (a) Before chr. $\frac{(3)}{(1)\,.\,(4)} \times 100.$	(b) After chr. $\frac{(3)}{(2)+(3)} \times 100.$	Recovery. %. $\frac{(2)+(3)}{(1)\,.\,(4)} \times 100.$	Total carotenoids. log. $\frac{Io}{I}$/ml. serum before chr.	β-carotene + neo-lycopene A + lycopene. % of total before chr.	Recovery. after chr.
0·060	0·020	3·00	11·2	13·9	81	0·150	47	93
0·053	0·019	2·85	11·8	13·6	86	0·144	50	96
0·074	0·019	4·00	12·7	13·8	93	0·129	49	92
0·072	0·018	4·00	11·3	12·6	90	0·128	53	99
0·080	0·020	4·00	12·8	13·9	92	0·129	51	96
0·069	0·017	4·00	11·9	14·5	82	0·157	44	96
0·064	0·016	4·00	9·8	11·6	85	0·156	46	97
0·069	0·017	4·00	10·1	10·4	97	0·156	46	99

on the Levels of Vitamin A Alcohol and Esters.

3. One single dose of 48,000 i.u. vitamin A alcohol.
4. One single dose of 48,000 i.u. vitamin A alcohol (different preparation).

Vitamin A esters. log. $\frac{Io}{I}$. Found. (3)	per ml. serum.	Amount of serum used for extraction. ml. (4)	Proportion of esters. % of total. (a) Before chr. $\frac{(3)}{(1)\,.\,(4)} \times 100.$	(b) After chr. $\frac{(3)}{(2)+(3)} \times 100.$	Recovery. %. $\frac{(2)+(3)}{(1)\,.\,(4)} \times 100.$	Total carotenoid. log. $\frac{Io}{I}$/ml. serum before chr.	β-carotene + neo-lycopane A + elycopen. % of total before chr.	Recovery after chr. %.
0·697	0·249	2·80	59·7	63·9	93	0·169	41	101
1·36	0·380	3·58	69·2	74·5	93	0·178	43	93
0·083	0·017	5·00	10·7	11·2	97	0·204	51	92
0·047	0·016	3·00	10·0	10·5	95	0·162	45	100
0·105	0·035	3·00	18·5	20·6	90	0·161	45	100
—	—	—	—	—	—	—	—	—
0·046	0·015	3·00	11·8	12·1	98	0·215	47	99
0·364	0·121	3·00	46·5	51·2	91	0·223	45	98
0·129	0·043	3·00	22·2	24·8	90	0·216	48	100
0·059	0·020	3·00	12·8	12·8	100	0·210	55	97
0·496	0·165	3·00	50·2	54·0	93	0·221	56	96
0·521	0·174	3·00	54.2	54·4	100	0·211	55	95
0·112	0·037	3·00	20·3	20·6	98	0·212	56	97

Raised vitamin A levels.—Three conditions in which the vitamin A level is known to be raised were studied: (*a*) after parturition (Abt, Aron, Bundesen. Delaney, Farmer, Greenebaum, Wenger and White, 1943); (*b*) after administration of alcohol (Clausen *et al.*, 1941); and (*c*) after administration of vitamin A,

(a) *Table VI* shows the changes in the levels of vitamin A at delivery. The total vitamin A rose after delivery in two cases. The vitamin A alcohol fraction rose by approximately the same amount, while the ester fractions remained unchanged. Case R. B—, from whom no sample had been obtained before delivery, was included because the analyses indicate that in this particular case no change in the total or esterified vitamin A occurred in a period of 5 days after delivery.

(b) *Table VII* shows the results of analyses of samples obtained before and after doses of alcoholic drink containing 20, 40 and about 60 ml. ethanol. The two smaller doses were taken within 3 mins. as a 20 per cent solution of ethanol in water. The larger dose was taken in the form of whisky diluted to a concentration of ethanol of about 15 per cent within 20 mins. Significant increases in the total vitamin A level were obtained with the two larger doses, and in both cases these were paralleled by increases in the vitamin A alcohol fraction. In Experiment I no sample had been withdrawn immediately before the dose of ethanol. The difference in the total vitamin A between the two samples taken after the dose was accounted for again by a change mainly in the level of vitamin A alcohol. In all cases the levels of the vitamin A esters remained steady. Symptoms of alcohol intoxication were very slight in both subjects.

(c) *Table VIII* shows the effects of the oral administration of vitamin A. In the first experiment, in which about 100,000 I.U. vitamin A were given as halibut liver oil which contains mainly vitamin A esters, the level of vitamin A alcohol remained nearly constant throughout, while the rise occurred in the ester fraction. The levels observed in the second experiment, in which about 5000 i.u. of vitamin A were given as the free alcohol (prepared by chromatographic separation from the unsaponifiable fraction of halibut liver oil and dissolved in about 1 oz. of butter), show that under these conditions also the major change had occurred in the ester fraction.

The total vitamin A rose by 21 per cent, while no change could be detected in the vitamin A alcohol level. The difference in the recoveries of vitamin A after chromatography, however, equivalent to an extinction of about 0·030/3 ml. serum, may have been caused by errors in the estimation of the total vitamin A, or by a difference in the losses on chromatography or both. In the latter cases a change in the vitamin A alcohol level might have been missed. But even if the difference in recovery is assumed to be wholly due to vitamin A alcohol, it can account for only 7 per cent increase in the total vitamin A.

In two subjects 48,000 i.u. vitamin A alcohol (freshly prepared and dissolved in 1 oz. of butter) produced a large increase in the levels of total vitamin A (Expts. 3 and 4). In both cases the rise in the ester levels accounted for 95 per cent of the total rise at its maximum, if differences in recoveries are ascribed to destruction on chromatography of both alcohol and esters in proportion to their respective concentrations. If only vitamin A alcohol is assumed to have been destroyed on chromatography, the percentages are 81 for Experiment 3 and 83 and 93 for Experiment 4. The small rise found in the vitamin A alcohol fraction appeared to have occurred in both cases later than the rise in the ester fraction, if the

changes in the protein concentration are used to correct for the changes in the vitamin A concentration due to dilution or concentration of the blood. Whether this rise is due to a direct passage into the blood of vitamin A alcohol or to a response of the liver is being investigated.

DISCUSSION.

The vitamin A esters make up a small proportion only of the total vitamin A in serum. In subjects in whom an endogenous increase in the vitamin A level is produced the increase is due to that in the vitamin A alcohol level. On the other hand, when vitamin A, esters or alcohol, is given by mouth, the increase occurs in the ester fraction. This confirms the view that re-esterification takes place during or soon after absorption (Drummond *et al.*, 1935). Since the store of vitamin A in the liver consists mainly of vitamin A esters, there is either a mechanism which retains the vitamin A esters in the liver and releases only vitamin A present in the alcohol form, or the esters are split before being released.

Ethanol in doses up to 60 ml. raised the vitamin A level by 10–15 per cent. After doses of 40 and 20 ml. the levels remained slightly raised or fell again within a few hours. The level was still increasing 2½ hours after the 60 ml, dose. These findings are in agreement with those of Clausen *et al*, (1941), and in conjunction with Yudkin's experiments (1941) they indicate that the minimal dose that shows a significant effect lies between 20 and 40 ml. ethanol.

SUMMARY.

A small proportion of the vitamin A in human serum is in the form of esters.

In 14 normal sera the proportion of esters to total vitamin A ranged from 10 to 17 per cent.

Vitamin A is released from the body stores after delivery and under the effects of ethanol in the form of vitamin A alcohol.

When the serum level of vitamin A was raised by oral administration of vitamin A as halibut liver oil or as vitamin A alcohol, the increase occurred in the vitamin A ester fraction of the serum. Doses of 48,000 i u vitamin A alcohol caused in addition a small delayed rise of the order of 10 or 15 per cent in the vitamin A alcohol fraction.

A method for the determination of vitamin A esters in serum is described.

Much help has been obtained from discussion and co-operation with members of the National Institute for Research in Dairying, Shinfield particularly with Dr. S. Y. Thompson. The author also wishes to express his thanks to Vitamins Ltd. for a grant providing for assistance and for a sample of β-carotene ; to Dr. M. E. Mawson, of the N.I.R.D., Shinfield, for a sample of liver oil concentrate of known biological activity; and to the Staff of the Department of Obstetrics of the London Hospital for facilities for taking blood samples.

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ELECTRON-MICROSCOPY OF VIRUSES: I. STATE OF AGGREGATION OF TOBACCO MOSAIC VIRUS.

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BAWDEN and Pirie (1937) noted that the properties of purified preparations of tobacco mosaic virus differed in a number of ways from those of virus as it occurs in freshly extracted sap. They suggested that virus particles were aggregating linearly during the course of purification, and that the greatly elongated rods characteristic of purified preparations might be artefacts. Since then evidence that purified perparations are heterogeneous in particle size and that preparations made by different methods may contain particles of very diverse lengths has come from studies of various physical properties. Such estimates of the length of virus particles in purified preparations have ranged from less than 300 mμ. to more than 1400 mμ. (*cf.* review by Pirie, 1945). The electron microscope provides an attractive direct* method of measuring particle size, and has already been used by several workers. It has confirmed that particles are more or less uniform in width and vary considerably in length, but there has been much difference of opinion as to the size of the basic units that aggregate to produce the long rods.

* In fact it is little more " direct " to use a lens to combine a diffraction pattern into an image which is then measured, than to record and measure the diffraction pattern itself. However, it is more straightforward to relate two lengths, that of the image to that of the object by a simple ratio (the magnification), than to deduce a length from some other physical quantity of more complicated dimensions. It is with these considerations in mind that the word " direct " is used here.

Kausche, Pfankuch and Ruska (1939) made the first measurements and gave a length of 150 to 300 mμ. Melchers, Schramm, Trurnit and Friedrich-Freska (1940) reported lengths of 140 and 190 mμ. Schramm (1943) also refers to electron micrograms* of tobacco mosaic virus as showing particle lengths of "about 200 mμ." Stanley and Anderson (1941) concluded from " measurements of the lengths of all of the 58 particles in two selected fields " that a unit having a length of 280 mμ. predominated. They concluded that the difference between their measurements and those of Melchers *et al.* was due to a difference in the virus strains. Lauffer (1944), from measurements on two prints from two preparations, concluded that the particle length was approximately 270 mμ., but that particles of approximately double this length were also present. However, Frampton (1942) and Rawlins (1942) did not consider the conclusions of Stanley and Anderson valid. Frampton, from measurements of the published electron-micrograms of Stanley and Anderson, concluded that they showed the particles to be made up by the aggregation of units 37 mμ. long. Rawlins did not admit even this regularity, and on the basis of new measurements on the same micrograms, correctly maintained that there are no statistically significant regularities at all. However, in a later publication, Rawlins, Roberts and Utrech (1946) claim that the majority of particles are about 300 mμ. in length, that longer particles are formed by aggregation of these, and that smaller units are due to breaking of rods during the drying which is necessary before examination in the electron microscope.

Light has recently been shed upon this somewhat chaotic situation by the demonstration (Bawden and Pirie, 1945) that it is possible to prepare tobacco mosaic virus in different states of aggregation, and that the particle length of any given preparation is largely a function of its previous treatment. They arrived at their conclusions on the basis of serological, ultracentrifugation and birefringence data, and their conclusions are fully confirmed by the electron microscope data presented in this paper.

EXPERIMENTAL.

Material.

Bawden and Pirie (1945) found that the conditions obtaining in expressed sap led to aggregation of tobacco mosaic virus particles. By using suitable methods, involving freezing and washing the leaves before mincing, they were able to extract virus while causing little aggregation. The virus in such extracts, however, was still far from homogeneous, and could be separated by ultracentrifugation into fractions with different properties. There is a steady gradation in properties from one fraction to another, but their method separates four fractions. One (D) sediments and compacts at 17,000 r.p.m. (R.C.F. 17,000), another (A) sediments but does not compact at 40,000 r.p.m. (R.C.F. 80,000), whilst B and C are intermediate. Fractionations were made of material which had been extracted by mincing and also from material subsequently passed through a triple-roller mill. These will be referred to as mincer and mill extracts respectively. We received for electron microscope examination various fractions prepared from leaves of tobacco (var. White Burley) infected with a mild strain of

* Chilton (1944) has suggested that it is convenient to restrict the terminations " -scope," " -graph," and " -gram " to the viewing instrument, the recording instrument, and the record respectively, as is usual for spectroscope, spectrograph and spectrogram. This terminology is adhered to here so that the more usual " electron-micrograph " becomes " electron-microgram."

tobacco mosaic virus, and also some of these same fractions after they had been subjected to various treatments. Purified preparations of potato X (Bawden and Pirie, 1938*a*), tomato bushy stunt (Bawden and Pirie, 1938*b*) and tobacco necrosis (potato culture) viruses (Bawden and Pirie, 1942) were available for comparison. The same tobacco mosaic strain and potato virus X were also examined in sap from tobacco plants (var. White Burley) infected with these viruses. The sap was prepared by freezing and centrifuging to remove chromoproteins.

Methods.

Specimens for comparison with each other were prepared all at one time and as soon as received to obviate as far as possible changes which may occur in the test-tube. A drop of dilute solution was placed on a membrane-covered metal grid and was rapidly dried in a vacuum desiccator. Because of drying and edge effects (see p. 337) the drops were usually placed asymmetrically so that the edge of the drop crossed the area to be examined in the microscope. Such drops were flat and about 2 mm. in diameter. Rather larger symmetrically placed drops were also employed, and microdrops covering an area 20–50μ in diameter have been examined. Concentration of virus in the suspension was varied from 1 in 10^4 to 1 in 10^6, according to the expected particle size, and higher concentrations were used for microdrops.

The membranes used in this work have been, in most cases, collodion stripped from glass. In our experience it is impossible to make really satisfactory membranes by spreading on water. When such membranes have once been in the microscope or have been kept in either a dry or a damp atmosphere for some days they can no longer be exposed to the beam without considerable risk of disintegration. Stripping from glass produces much more uniform and robust membranes, which can be stored indefinitely without any special precautions other than to keep them dust-free and can be placed in the microscope a number of times. Microscope slides (3 in. $\times$ 1 in.), selected for even surface, are prepared by boiling in cleaning fluid, washing under the tap and then in grease-free distilled water and draining. They are then polished with MgO, moistened with alcohol on a cotton-wool swab, the MgO removed with a clean dry cotton-wool swab, and finally by washing in dilute HCl followed by distilled water. The water is replaced by alcohol, which is finally removed by flaming. On such a clean and polished surface both aqueous and organic solutions will spread with ease, and from it membranes are readily removed.

Collodion (Schering celloidin is satisfactory) is dissolved in analar amyl acetate. 0·25 per cent solutions are most generally satisfactory. One surface of a prepared slide is flooded with solution, the surplus is drained off and the slide is stood on end on filter-paper in a desiccator at atmospheric pressure until the solvent has evaporated. Drying in a desiccator is useful to prevent " blooming " of the film. Slides bearing dried membranes can be stored indefinitely in Coplin jars at this stage. The membrane thus produced is wedge-shaped and is approximately 10 mμ. thick about the middle of the slide and, provided the glass surface was good, it is free from holes. The film is divided into $\frac{1}{2}$ cm. squares by scoring with a needle and the slide is lowered gently into a dish of clean, grease-free distilled water at an angle of approximately 30° to the horizontal. The squares of membrane become detached and float on the surface.

The water surface is best prepared by filling a large crystallizing dish so full that the water meniscus stands above the brim, and then sweeping the excess off with a clean glass strip held in contact with the rim. In cold weather it may be advisable to heat the water to about 18° C. A mounting grid is placed on each square, and both are removed and dried and the specimen placed in the usual way.

Five to six specimens were made from each virus preparation and five to ten micrograms taken at random intervals over each specimen, no attempt being made to select the fields other than to try to ensure that virus was being recorded each time.

The electron microscope used was the R.C.A. Type EMB2. Most observations and photographs were made with accelerating potentials of 60kV, since Crook, Sheffield and Chilton (1945) have shown that higher contrast is obtained in the negatives at higher kilovoltages when tenuous specimens are being examined. Earlier observations before this information was available were made at 45 kV. The photographic plates used were the special Contrasty Lantern and Photomechanical of Messrs. Ilford Ltd., the latter being preferred. These were developed with rocking or brushing for 2½ mins. in Ilford ID16 and 2 mins. in Ilford ID13 developers respectively. The temperature of development was 18° C. We have found it advisable to aim at fairly dense negatives (density 1 to 2) in order to realize the maximum available contrast of the plates. Crook *et al.*(1945) have shown that whereas with light, the characteristic curves of the emulsions used here have a fairly sharp " foot " and begin to rise steeply from densities below 0·5, with electrons the " foot " is much more extended, and the curve does not reach its maximum slope until densities of 1·0 or more are reached.

For magnification calibration a diffraction grating replica has been used as primary standard. Earlier methods of calibration (R.C.A. Handbook, 1940) have used the light microscope and a stage micrometer as primary standard, and were limited by the necessity of having a small standard object, of assuming that the magnification of standard object and stage micrometer were identical, and by the necessity of having a good travelling microscope in order to measure the small light images with sufficient accuracy. The resolving power of the light microscope was also a limiting factor. Burton, Barnes and Rochow (1942) proposed the use of diffraction grating replicas as standard objects. They stated that shrinkage was constant and amounted only to 1½ per cent. However, in our experience, shrinkage or stretching may occur and either may vary in amount up to 1½ per cent. The alternative was to use the grating only as a mould, and measure the spacing on the actual replica. No first grade travelling microscope was available for this purpose, but we have been able to devise an optical method, using very simple apparatus.

The diffraction grating was a 14,400 lines/inch concave nickel grating kindly placed at our disposal by the National Physical Laboratory. We have found that the most suitable method of cleaning is to swab gently with a Na lauryl sulphate solution, wash in distilled water followed by alcohol and to dry in a desiccator. Replicas were made by flooding the grating with 0·5 per cent formvar solution in water-free dioxan, and after evaporation of the solvent, stripping on to water as described above. The replicas were transferred to the highly polished side of copper grids of 200/inch mesh in the usual way. The grids were mounted in a holder on a student's type spectrometer and adjusted in the usual

way (Watson, 1930). If no Gaussian eyepiece is available, a substitute can easily be made by supporting a microscope cover-glass at an angle of 45° over the normal eyepiece with a piece of soft wax. The light source was an 80 watt Hg-arc (G.E.C. " Osira " electric discharge lamp with the outer bulb removed), although a higher power would have been better. The arc was focused on the slit of the spectrometer with a short focal length lens—a substage condenser (Leitz aplanatic N.A.1.4) with the top lens removed worked very well—so that it just covered the slit. Sufficient light was thus obtained for the collimated beam to be stopped down to 1/2 mm. diameter and still yield visible 1st and 2nd order images of the Hg green and yellow lines, provided the room was dark. The beam was conveniently stopped down by supporting with a clamp the iris diaphragm from a microscope substage assembly. Measurements of the diffraction angles of the Hg green and yellow lines thus gave directly the average replica spacing over the area covered by the R.C.A. type B2 microscope. Other types which have a larger field would not restrict the lighting conditions so much. In practice it was found that the diffracted images were not single, but were a narrow pattern of lines due to variation in replica line spacing and to slight buckles in the surface of the grid. However, in the replicas selected for use, the total width of this pattern corresponded to much less than 1 per cent of the average grating spacing.

As with other standard objects, its comparatively large size necessitated the taking of the initial calibration pictures with the object in the " low magnification " position. For internal calibration, using this initial picture as standard, we have found diatom fragments to be most suitable. For the step across from the " low " to " high magnification " positions fragments were selected which had holes just discernible in the " low " position, and for internal calibration in the " high " position those with holes less than 0·1μ diameter in convenient positions. The centres of the images of these holes on the negative formed convenient points on which to set the cross-wires of a travelling microscope. Suitable fragments are easily obtained by shaking up a little kieselguhr in water and standing overnight. The very fine particles are removed by pouring off the remaining suspension. The sediment is again shaken up with water, and after standing two or three hours yields a suspension of particles of a convenient size. It can be poured off and kept, the addition of a drop of chloroform discouraging bacterial contamination.

As this paper deals only with the qualitative aspects of the problem, and as lens aberrations make different corrections necessary for particles in different positions, no attempt has been made to give exact magnification data for each microgram. The figures given are therefore only approximate (accuracy $\pm$ 20 per cent). In a later paper quantitative data will be presented in detail and the necessary corrections discussed.

RESULTS.

Bawden and Pirie (1945) have shown that, passing from their A to their D preparations, there is a continual gradation in properties. The sedimentation constant increases, the double refraction of flow increases, the rate of precipitation with antiserum increases and the tendency to form a zone of antigen excess decreases. At the same time the character of the precipitate changes from

that typical of somatic to that typical of flagellar antigens. As they have indicated, this can be most satisfactorily interpreted as an increase of particle length.

Fig. 3 to 6 are electron micrograms of a set of preparations from mill extract. They show that there is a considerable increase in particle length on passing from one end of the series to the other. Fig. 3 is a typical field from an A preparation. Here no rods can be distinguished, although a few particles may be asymmetric. Indeed, so untypical of tobacco mosaic virus as it is generally recognized are micrograms of A-type preparations, that it is impossible to distinguish them at a glance from tomato bushy stunt and tobacco necrosis viruses, which are shown at the same magnification in Fig. 17 and 18. Critical examination shows the sizes in descending order to be bushy stunt, tobacco necrosis, tobacco mosaic A-type preparation. This is in keeping with the fact that their sedimentation constants are 132, 112 and approximately 30×10^{-13}.

In the B preparation (Fig. 4) rods are undoubtedly present, although these are very short, and there is a considerable proportion of the A-type particles. In C-type preparations (Fig. 5) the majority of particles are small rods, some long rods are present, and the proportion of A-type particles is very much reduced. Finally, in D-type preparations long rods predominate, whilst A-type particles are present only in small proportions, although not entirely absent. Indeed, even in preparations containing extremely long particles, such as Fig. 8, it is possible to find a few of what appear to be A-type particles.

Although it is possible to obtain A-type preparations from mincer-extracts which give micrograms identical with Fig. 3, in general the virus prepared from these is in a more highly aggregated state than that obtained from mill extracts, as can be seen by a comparison of Fig. 1 and 2 with Fig. 5 and 6. In these micrograms (Fig. 1 and 2) the C-type contains some particles longer than those shown in the D-type. This is possible for two reasons. In the first place the micrograms were prepared from fractionations made on two separate occasions, the bulk from which Fig. 1 was separated being on the average more aggregated than that from which Fig. 2 was prepared. In the second place, although Fig. 1 contains single particles which are longer than many in Fig. 2, it also contains many more which are shorter, i.e. its average particle length is less. This pair of micrograms illustrates very well the extreme difficulty of preparing homogeneous tobacco mosaic virus, and the danger of assuming that because two preparations have been made from similar starting material, by similar preparative manipulations, they are necessarily identical in so far as particle size, particle size distribution and properties which depend on these are concerned.

Bawden and Pirie (1945) showed that one of the most effective means of causing aggregation was incubation with phosphate and trypsin. Fig. 7 shows the effect of incubating a B-type preparation with phosphate and trypsin (2 g./l of virus, 2 g./l phosphate, pH 6·0, and 0·13 g./l of dialysed B.D.H. trypsin, 2 hours at 37° C.). The average particle length has increased considerably. The ultimate results of incubation with trypsin vary with the nature of the starting material and the exact conditions of incubation. Under favourable conditions the aggregation proceeds to very great particle lengths. Thus Fig. 8 and 9, of trypsin incubated D-type preparations, show particles 100 or more times as long as they are wide. In these preparations it is very difficult to estimate the particle size because of the pronounced tendency to form " mats " of the type of Fig. 9.

The largest single particles that we have observed passed right across the field of view and must have been more than 5μ long.

As Bawden and Pirie have indicated, the smaller fractions are unstable and pass readily into forms with larger particles, the change being accelerated by the presence of salts and other materials present in sap. The extreme ease with which aggregation occurs is illustrated by Fig. 10. This is an almost salt-free B-type preparation, initially giving micrograms indistinguishable from Fig. 4, but which had been kept for 3 weeks in the refrigerator at 4° C. Considerable aggregation has occurred even under these conditions. Subsequent treatment

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DESCRIPTION OF PLATES.

All figures are electron micrograms taken with an R.C.A. (type EMB. 2) instrument. Fig. 1–12 were made with an accelerating potential of 60 kV., and the remainder at 45 kV. Magnifications on the negatives of the former were approximately 13,000 ×, and of the latter about 16,000 ×. Micrograms were enlarged photographically either 3 × or 2½ ×, and have been reduced in reproduction to give a magnification of about 26,000 ×. It should be emphasized that these figures are approximate only. In determining the size of any particular particle, we find it necessary to correct for errors which may amount to as much as ± 20 per cent, and corrections vary with the angular position of the particle. These will be discussed in a subsequent paper.

Tobacco Mosaic Virus.

Preparations shown in Fig. 1–6 were separated by fractionation in the ultracentrifuge and have received no other treatment. Type A sedimented most slowly, D most rapidly, and B and C were intermediate.

FIG. 1.—C-type from mincer extract.

FIG. 2.—D-type from mincer extract.

FIG. 3.—A-type from mill extract.

FIG. 4.—B-type from mill extract.

FIG. 5.—C-type from mill extract.

FIG. 6.—D-type from mill extract.

FIG. 7.—Mill extract C after treatment with trypsin and phosphate (*cf.* Fig. 5).

FIG. 8 and 9.—Mill extract D treated with trypsin and phosphate (two fields from the same preparation).

FIG. 10.—Mill extract B kept 3 weeks at 4° C. (*cf.* Fig. 4).

FIG. 11.—Mill extract B kept 3 weeks at 4° C. and then treated with phosphate (*cf.* Fig. 10).

FIG. 12.—An old and much aggregated preparation evaporated to dryness and re-wetted 3 times.

FIG. 13.—Crude sap. Rods are visible among small particles of normal plant constituent.

FIG. 14.—Crude sap (as Fig. 13) heated to 70° C. for 10 min.

Potato X Virus.

FIG. 15.—Crude sap. Rods are seen amongst the normal plant constituents.

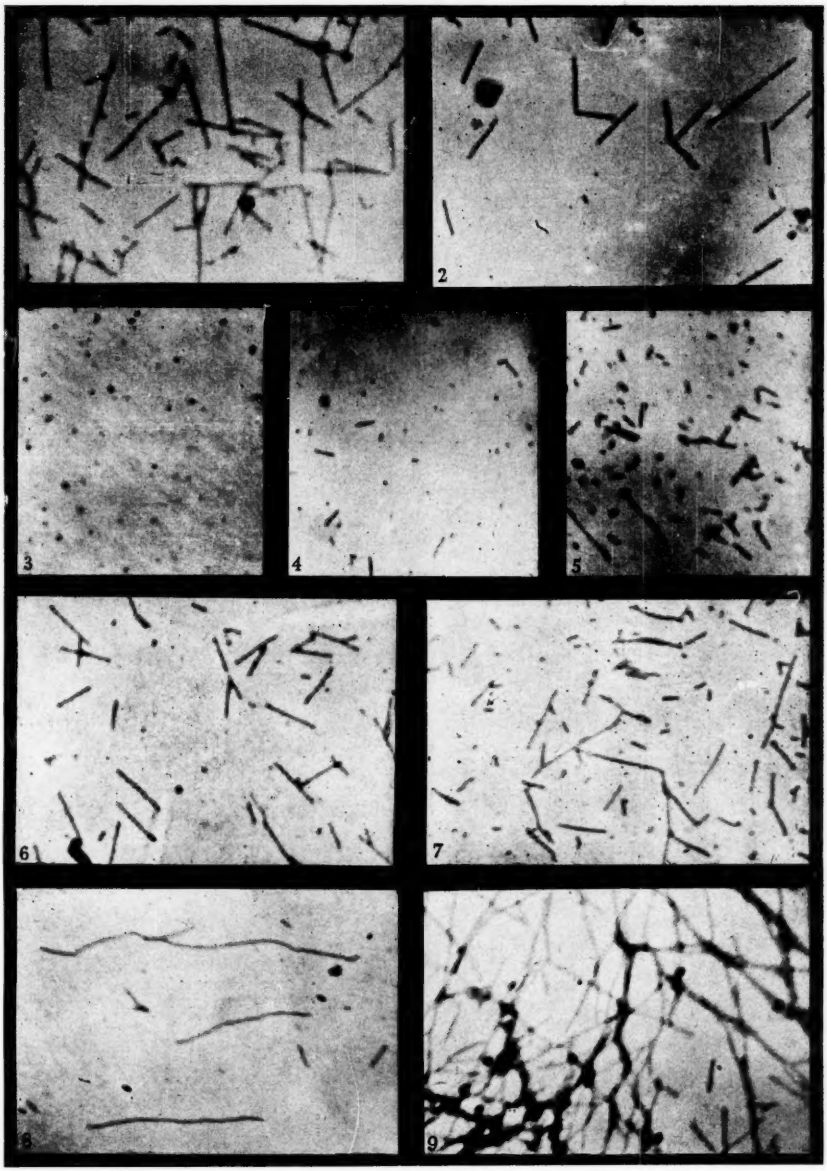
FIG. 16.—Purified preparation.

Tobacco Necrosis Virus.

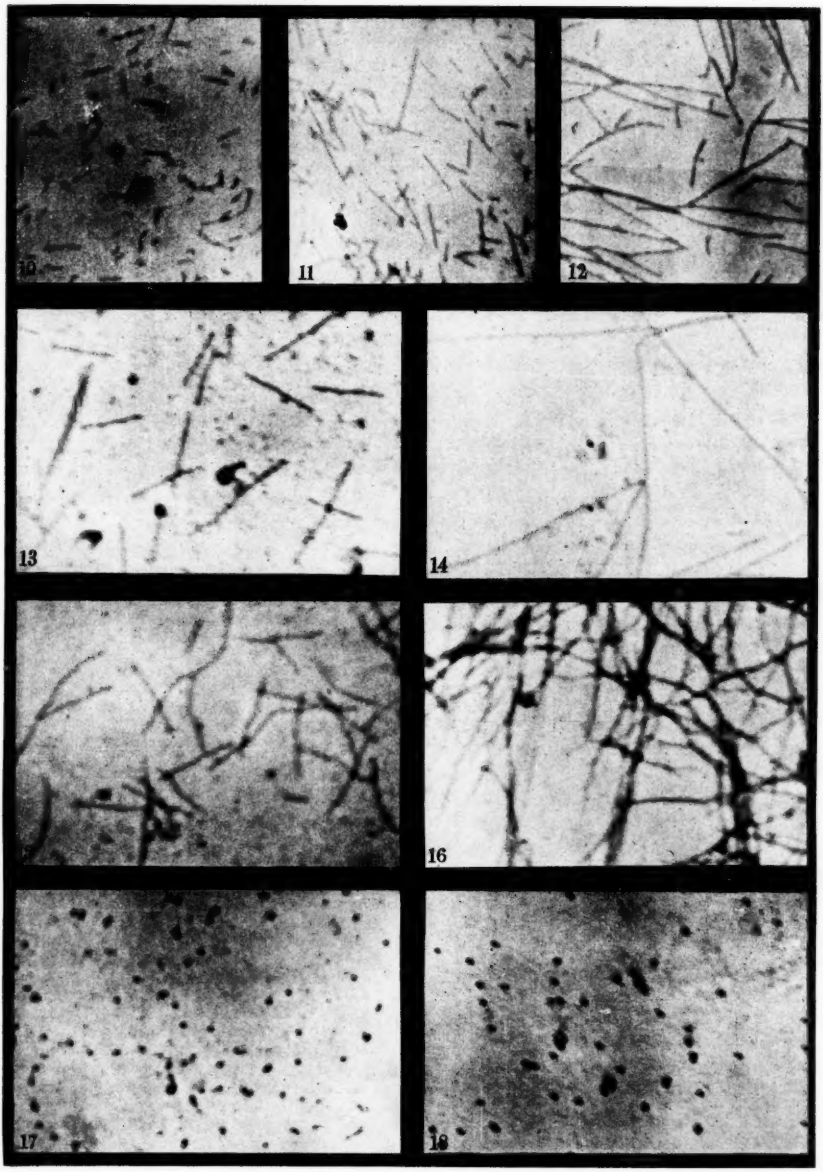
FIG. 17.—A purified preparation of mixed strains.

Tomato Bushy Stunt Virus.

FIG. 18.—Purified preparation.



Crook and Sheffield.



Crook and Sheffield.

with phosphate (2 g./1, pH 6·0, 2 hours at 37° C.) causes aggregation to proceed further to the stage shown in Fig. 11.

One difficulty of interpreting electron micrograms arises from the lack of precise knowledge of the effect of drying on the virus particles. Bawden and Pirie (1937) have shown that drying inactivates the virus and reduces the anisotropy of flow. To try and arrive at some estimate of the effect of drying, a mixed batch of old trypsin-treated preparations was allowed to evaporate on the laboratory bench, the dried material taken up in water and the process repeated twice more. Thus, including the drying incidental to the making of the microscope specimen, this material had been dried four times in all compared with once for a normal preparation. Fig. 12 shows that it has suffered no serious disaggregation. Many small particles are present, but they are not sufficiently increased in number, nor the larger ones sufficiently diminished for there to be any confusion with, say, an A-type preparation.

Aggregation of a similar type occurs not only in purified preparations, but in crude sap. Indeed, conditions in sap are such as to favour aggregation, and treatments applied in this medium lead to a large degree of aggregation. For example, Fig. 13 shows particles of tobacco mosaic virus as they appear in crude sap. Small particles may be difficult to see because of the background of normal leaf material, but the larger particles are sufficiently distinctive to be easily identified. The effect of heating this preparation to 70° C. for 10 mins. is shown in Fig. 14. Here the rods have elongated so greatly that they extend across the whole field.

That this linear aggregation of particles is not confined to tobacco mosaic virus but occurs in at least one other virus with rod-shaped particles is illustrated in Fig. 15 and 16. The former is a microgram of potato virus X as it occurs in crude tobacco sap. The latter shows the state of increased aggregation which may result when the virus is purified by certain methods.

Fig. 17 and 18, tobacco necrosis and tomato bushy stunt viruses, are included, mainly for comparison with A-type preparations of tobacco mosaic virus. However, they do show the character of these virus particles very well, possibly better than other micrograms so far published. Whether the slight tendency to asphericity noticeable in these micrograms, particularly in that of tobacco necrosis, is real or whether it is due to clumping of the particles, distortion during drying, or some defect in the microscope optical system cannot as yet be ascertained.

DISCUSSION.

The results described here show that there is no basis for assigning a " length " to " the molecule " of tobacco mosaic virus from electron microscope measurements as has been attempted by several workers. Even in a single preparation, the length of the individual particles varies considerably, and by varying the conditions under which the preparation is made the average size of the particles can be varied from those shown in Fig. 3 to the " mat " of Fig. 9. The present data also show that Markham, Smith and Lea (1942) were in error when they concluded that " an estimate of size can be given with some confidence," although the data available at that time were in some measure self-consistent.

Stanley and Anderson (1941) also had variable length particles to deal with.

Thus, their data were obtained by measuring "all of the 58 particles in two selected fields." The selection would naturally be to obtain as many likely looking particles as possible in a field, i.e. to select uniform fields. Yet even under these conditions, on a weight basis only 50 per cent of the material occurred as particles of 280 mμ., and their histogram shows that fewer than half the particles were of this length. Frampton (1942) has pointed out that there are particles as short as 37 mμ. in their micrograms, and suggested that these might be the basic units from which the larger rods were built up. However, the A-type preparations shown here obviously have particles smaller than this—many being at least as short as the diameter of the rods (15 mμ.). This is in agreement with Rawlins' (1942) criticisms of Frampton when he pointed out that the regularities in Frampton's measurements were not sufficient for any conclusion to be drawn about the size of a basic unit. However, there was no basis for Rawlins' argument that because of these irregularities in length he was dealing, not with a "molecule," but an "organism." If this argument were followed to its conclusion we should be endowing with "life" the particles of many a thermoplastic polymer merely because they were not uniform in length.

Stanley and Anderson, in discussing the small particles which appear in their micrograms, concluded that their true nature and significance was unknown, but implied that they might have been produced at the time the specimen was mounted, since there was no evidence for their presence from ultracentrifugal analysis. Rawlins *et al.* (1946) made a similar suggestion. From the data presented here it can be seen that although drying may cause a small amount of disaggregation (Fig. 12), many preparations contain exclusively short particles. We therefore conclude that short particles occurring in long particle preparations are not merely artefacts or breakdown products, but normally occurring constituents. The relationship between these particles and the longer rods has been fully discussed by Bawden and Pirie (1945).

It is unlikely that the differences between previous results and those reported here are due to any basic differences between the virus strains. Stanley and Anderson (1941) concluded from the difference between their measurements and those of Melchers, Schramm, Trurnit and Friedrich-Freska (1940) that different virus strains have different particle lengths. That postulate appears to be unnecessary, since the particle length of a preparation from a single strain depends so closely upon its previous history that there is adequate scope for the observed variations within it. Now that this variation has been disclosed, a large accumulation of data will be necessary before any variation between strains can be adequately demonstrated.

As all the micrograms of tobacco mosaic virus so far published show considerable variation in the size of the particles, it is curious that this variation has been largely ignored. Such neglect is probably due partly to the preconceived idea that viruses are macromolecules and therefore should be uniform, and partly because most workers have not examined a sufficient number of micrograms to emphasize the variability. Thus Stanley and Anderson (1941) base their findings on examination of two selected fields from a single preparation, and Lauffer (1944) on one microgram from each of two preparations. Before such measurements are valid two conditions must be fulfilled: the field photographed must be representative of the specimen, and the specimen itself representative of the preparation as a whole.

The former of these conditions is fulfilled by making a number of records of each specimen. However, the latter condition is much more difficult to fulfil satisfactorily because of several phenomena connected with the drying of virus solutions. Dilutions of the order of 1 in 10^5 or 10^6 are necessary to obtain particles sufficiently dispersed to be able to distinguish individuals. At these dilutions, a considerable fraction of the virus is in the form of a surface film which is likely to contain a different ratio of large to small particles than that in the bulk phase. If, by chance, portions of this surface film are examined, a spurious ratio of small to large particles may be recorded. Again, even at dilutions of 1 in 10^6, virus tends to move to the outer fringe of the drop during drying, forming a " mat " too dense for the electron beam to penetrate, while the area occupied by the centre of the drop is left almost barren of particles. It is not known whether this movement is due to the surface film or whether it affects small and large particles equally, and in any event, it often makes difficult the preparation of a specimen with concentrations of virus at the centre of the field optimum for viewing. In the present work we have attempted to overcome these disabilities by using both centrally placed drops and drops placed asymetrically so that the region of " mat " formation crosses the field of view. We have thus been able to examine both regions. While we do not claim that this is an adequate solution of the problem, it does ensure that the virus is examined under a fairly wide range of conditions. The use of a sufficient number of preparations and an adequate number of photographs from each improves the chances of obtaining representative micrograms. The present material has been selected as being the most representative set from nearly 700 negatives made from 29 different preparations of tobacco mosaic virus.

In certain of the preparations (Fig. 7, 8) apparently continuous rods may not be straight, but may change their direction abruptly several times. It seems probable that these discontinuities are the points at which shorter particles have joined, and may correspond to those shown in the excellent shadow-cast micrograms of Williams, Robley and Wyckoff (1945). Occasionally an actual break in the rod is visible (Fig. 6).

The data presented here include no evidence regarding a possible basic unit from which the particles are built nor of the size of such a unit. From an examination of micrograms of A-type preparations, however, it can be concluded that such a unit, if it exists, is small, possibly too small even to be seen or measured in the electron microscope. This follows from the fact that such preparations contain material of length at least as short as the diameter of the rods. Nor is evidence given here as to whether the aggregation proceeds continuously or discontinuously, i.e. whether or not there are aggregates of certain lengths which are more stable than others. It seems quite likely that this is so, and indeed it would explain the frequency with which a length of approximately 300 mμ. has occurred in measurements of tobacco mosaic virus. It is hoped shortly to present evidence on both these points, together with some numerical data on the effect of preparative history upon particle length.

In conclusion, it is worth emphasizing that since we have demonstrated linear aggregation in at least one other virus with rod-shaped particles, namely, potato X virus, all figures for particle size of other rod-shaped viruses (e.g. poliomyelitis) must remain suspect until it is proved that they are not being invalidated by the same phenomenon.

SUMMARY.

Preparations of purified tobacco mosaic virus separated according to Bawden and Pirie's (1945) method of differential ultracentrifugation have been examined in the electron microscope. Micrograms of the most slowly sedimenting fraction (A) contain particles most of which are no longer than their diameter (15 mμ.), and these particles are compared with the spherical viruses, tobacco necrosis and tomato bushy stunt. As sedimentation rate increases (types B–D) the specimens contain rods of increasing length. Micrograms of crude sap show rods mixed with other plant constituents. Keeping the virus for 3 weeks at 4° C. causes some particles to aggregate, and aggregation proceeds further during treatment with phosphate. Heating under certain conditions to 60° C. or more or incubating at 37° C. with trypsin produces many rods longer than 5 μ. Drying causes no serious disaggregation.

Particle length varies also in potato X virus.

In no preparation of a rod-shaped virus were the particles all of uniform length, and in tobacco mosaic virus some particles of the A-type were always present. All evidence indicates that average rod length is dependent on preparative treatment.

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CHEMICAL CHANGES IN MUSCLE DURING AND AFTER ISCHAEMIA.

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THE development of clinical shock after muscle trauma or on release of a tourniquet after prolonged ischaemia has, in general, been ascribed either to a decrease in blood volume by the loss of plasma into the traumatized area, or to the absorption from this area of some substance with a toxic effect. These explanations are not mutually exclusive, and may have a common basis in an altered permeability of the tissue brought about by anoxia. It is recognized that the leakage of plasma protein is due to an alteration in the permeability of the capillary endothelium, but in view of the work of Rosenthal and his colleagues, which has recently been summarized by Rosenthal and Tabor (1945), the permeability of the muscle fibre in these conditions becomes of great interest. These authors showed that in various forms of experimental shock there was a great loss of sodium, drawn from the blood and other tissues, into the damaged area, and that the lives of the animals could be saved by the administration of sodium salts soon after injury. While the extracellular tissue space is accepted as being in diffusion equilibrium with the plasma, it is well known that the muscle fibre displays a highly selective ion permeability (Höber, 1945), the concentration of K being much higher and that of Na much lower than in the plasma. The values however approximate to those of the plasma on the death of the muscle (Hastings and Eichelberger, 1937). It is reasonable to suppose that the normal state of selective permeability of the fibre may be maintained by the expenditure of energy derived from the metabolism of carbohydrate, and that an increased permeability may be associated with some failure in this metabolic cycle. The chemical changes in muscle during and after ischaemia have been investigated from this point of view, taking in the first instance as an index of the level of the metabolic cycle the concentration of adenosinetriphosphate (A.T.P.), since this substance is a coenzyme of high potential energy necessary for the metabolism both of glycogen and glucose. The possible relationship between the A.T.P. level and other changes in the tissue which may directly determine the occurrence of shock has been considered by following also the rate and extent of entry of water and chloride into the muscle and the loss of pentose compounds and potassium from it.

EXPERIMENTS ON GUINEA-PIGS.

Methods.

Guinea-pigs of 400–500 g. were anaesthetized by injection of urethane (1·0 c.c./100 g.) or nembutal (0·07 c.c./100 g.), and ischaemia of one hind limb was produced by application of a screw-clamp round the upper thigh. At the desired time before or after release of the clamp the animals were killed by heart puncture,

and two samples of muscle excised immediately from the ischaemic and from the normal limb, one from the lower leg and one from the thigh. The muscle was wiped on filter-paper, and 1–2 g. of each sample sliced finely with scissors into tared vessels containing 10 c.c. of 10 per cent trichloroacetic acid. After re-weighing, the volume of acid was made up to 10 c.c./g. tissue and aliquots of the filtrate taken for analysis :

Adenosine-equivalent, assayed by the guinea-pig heart technique (Drury, Lutwak-Mann and Solandt, 1938) by comparison with adenosine, and expressed as such.

Pentose by colorimetric comparison with a known amount of A.T.P., after heating 10 mins at 100° with one volume 0·3 per cent orcinol, 0·1 per cent $FeCl_3$, in conc. HCl ; expressed as adenosine.

Phosphorus estimation (Martland and Robinson, 1926) ; total P, after wet ashing = ΔT.P. ; (ortho + creatine) P, by direct estimation $= \Delta P_{0\,min.}$; pyro-P after 7 min. hydrolysis in N HCl at 100° = $\Delta P_{7\,min.} - \Delta P_{0\,min.}$; organic P by subtraction = $\Delta T.P. - \Delta P_{7\,min.}$

Potassium (King, 1946) ; *chloride* by Volhard titration ; *creatine* (Eggleton, Elsden and Gough, 1943).

Sodium estimations were made on about a third of the samples after removal of phosphate with $Ca(OH)_2$ and colorimetric estimation (King, 1946) of the Zn uranyl acetate precipitate ; this method is not very satisfactory with muscle filtrates, but showed that the Na values were approximately parallel to the Cl values.

RESULTS.

Decomposition of A.T.P. during ischaemia.

After application of the clamp the pyro-P content of the ischaemic muscle fell gradually from the normal value of 0·36 mg./g. to a minimal value (0·04 mg./g.) in 5 hours. The rate of fall is rather slower, but the results are in general agreement with those of Bollmann and Flock (1944) for ischaemic rat muscle. These authors showed that the disappearance of pyro-P was preceded in the first hour of ischaemia by decomposition of phosphocreatine.

The adenosine-equivalent also decreased, parallel to the fall in pyro-P (Table I), indicating that the dephosphorylation of A.T.P. was accompanied by deamination of adenylic acid, with the formation of inosinic acid and orthophosphate. The content of pentose, K and total P was unchanged. The histamine content of the filtrates was not significantly different from the normal value.

TABLE I.—*Decomposition of A.T.P. in Guinea-pig Muscle During Ischaemia.*

Time after clamping (hrs.).	mg./g. fresh muscle ± S.E. (No. of samples).		
	Total P.	Pyro-P.	Adenosine-equivalent.
0	1·42 ± 0·02 (21)	0·36 ± 0·02 (21)	1·95 ± 0·10 (22)
1		0·32 ± 0·02 (4)	1·50 ± 0·25 (4)
2		0·27 ± 0·015 (4)	1·37 ± 0·20 (4)
3	1·45 ± 0·02 (22)	0·16 ± 0·01 (4)	1·12 ± 0·20 (4)
4		0·11 ± 0·015 (4)	0·62 ± 0·12 (4)
5		0·035 ± 0·012 (4)	0·35 ± 0·04 (4)

Changes in muscle on re-establishment of the circulation.

A clamp was applied to one thigh of animals under nembutal anaesthesia, and after a period of 3 or 5 hours' ischaemia the clamp was removed and the

animals sacrificed at times between ¼ and 48 hours after release of the clamp. Swelling of the whole limb and groin, apparently to the maximum for each degree of ischaemia, occurred generally within a few minutes after the circulation was re-established. Among the first group of animals treated in this way, ridging of the muscles occurred in a few at the site of the clamp and no swelling took place, but with practice in the adjustment of the clamp an effective ischaemia, as judged by the coldness and colour of the pad, was produced without permanent occlusion of the vessels. Apart from transient shivering on release of the clamp the animals in general appeared to suffer no ill-effect and only one out of the 30 animals treated died. Ischaemia of one hind limb has usually been found insufficient to produce fatal shock in small animals (Green, 1943 ; Bollman and Flock, 1944).

Samples of muscle were excised immediately after death and analysed as before. In the 3-hour ischaemia group the muscles were slightly swollen and damp, but otherwise normal in appearance. In the 5-hour ischaemia group the muscles were often boggy, with patchy haemorrhages throughout ; a few hours after release there was a considerable exudate containing protein and chloride, and at a later stage a gelatinous subcutaneous oedema.

TABLE II.—*Analyses of Muscle After Release from Ischaemia.*

Time after release (hrs.).	% H_2O.	mg./g. fresh muscle (corr. for increase in volume). Total P.	Pyro-P.	Adenosine-equivalent.	Pentose.	Cl.	K.
		Normal values.					
	76·1±0·9	1·42+0·02	0·36±0·02	2·0±0·1	2·1±0·04	0·54±0·02	4·3±0·09
		3 hrs. Ischaemia.					
0·5	78·1	1·51, 1·33	0·29, 0·29	1·6, 1·3	—	1·04, 1·02	4·0, 3·7
1	79·0	1·42, 1·47	0·29, 0·35	1·6, 1·3	2·3, 2·3	0·94, 0·75	3·7, 3·8
2	79·3	1·49, 1·48	0·35, 0·37	2·3, 2·3	—	1·18, 1·13	4·1, 4·0
	78·0	1·40, 1·40	0·30, 0·30	1·7, 1·7	2·1, 2·1	0·85, 0·60	4·0, 4·1
3	77·8	1·34, 1·27	0·22, 0·27	—	—	1·15, 1·27	—
6	77·8	1·47, 1·47	0·36, 0·36	2·7, 2·7	2·2, 2·2	1·10, 1·00	3·5, 3·2
12	78·9	1·29, 1·41	0·25, 0·29	0·9, 0·9	2·0, 2·3	1·50, 1·30	3·8, 5·1
	78·3	1·30, 1·53	0·29, 0·36	—	—	1·18, 0·93	—
	78·3	1·49, —	0·37, —	—	—	0·99, —	—
24	75·5	1·16, 1·24	0·27, 0·30	1·0, 1·0	1·7, 1·9	1·29, 0·83	3·4, 3·5
	78·3	1·45, 1·52	0·28, 0·30	—	—	1·12, 1·26	—
48	75·0	1·62, 1·59	0·39, 0·41	2·0, 2·0	2·4, 2·4	0·60, 0·46	—
	77·3	1·33, 1·47	0·38, 0·38	—	—	1·22, 1·01	4·5, 4·4
	77·2	1·64, 1·33	0·37, 0·33	—	—	0·71, 0·85	—
		5 hrs. Ischaemia.					
0·25	81·2	1·48, 1·51	0·32, 0·29	1·2, 1·2	1·6, 1·8	1·34, 1·16	4·0, 4·0
	82·0	1·47, 1·26	0·13, 0·12	0·5, 0·4	2·1, 1·8	1·05, 0·94	4·1, 3·5
1·5	82·4	1·33, 1·35	0·26, 0·22	1·1, 1·1	1·4, 1·4	1·69, 1·59	3·2, 3·2
	82·3	1·24, —	0·13, —	0·4, —	1·6, —	1·70, —	3·1, —
2	82·7	1·02, 1·14	0·15, 0·19	0·8, 0·9	1·2, 1·4	2·20, 2·30	2·9, 3·2
3	81·8	1·10, 0·80	0·08, 0·04	—	—	2·40, 2·70	—
	79·1	1·13, 1·25	0·02, 0·05	—	—	1·60, 1·64	—
6	81·0	1·15, 1·20	0·21, 0·23	1·4, 1·4	1·3, 1·4	2·00, 1·70	3·1, 3·1
12	79·7	1·17, 1·30	0·23, 0·28	0·7, 1·1	—	1·30, 1·00	3·3, 3·8
	81·7	0·60, 0·76	0·11, 0·13	0·5, 0·5	<0·6, <0·6.	3·40, 2·90	2·1, 2·6
	81·0	0·84, 1·31	0·17, 0·25	0·7, 0·6	0·7, 2·0	2·80, 1·40	2·0, 2·9
24	83·0	0·64, 0·45	0·11, 0·05	0·5, 0·3	<0·6, <0·6.	3·10, 3·60	2·2, 1·1
	82·1	0·52, 0·40	0·07, 0·05	—	—	2·40, 2·10	—
48	82.5	0·55, 0·44	0·05, 0·05	0·3, 0·2	<0·5, 0·5	3·50, 3·70	1·8, 2·0

The analytical figures have been corrected for the increase in volume due to the oedema by calculation from the water content, i.e. corrected value = mg./g. sample × per cent dry weight normal muscle/per cent dry weight sample muscle, so that a recorded decrease in a constituent represents a real loss from the muscle and not merely a dilution. It is assumed in this calculation that the dry weight of the whole muscle is not significantly changed, i.e. that any loss of muscle protein is offset by the entry of plasma protein.

The main results for two groups of animals released after 3 and 5 hours' ischaemia respectively are shown in Table II.

Pyro-P values.—In the 3-hour ischaemia group the pyro-P values at any time between ½ and 48 hours after release were higher than the ischaemic level, and the mean value for all the samples (0·32 ± 0·05 mg./g.) closely approached the

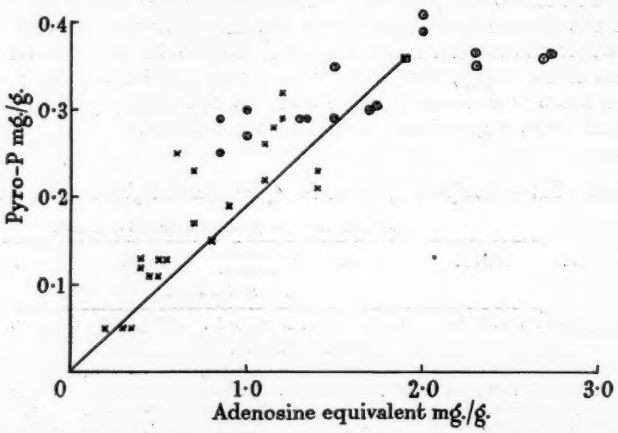

FIG. 1.—Correlation between pyro-phosphate and adenosine-equivalent in samples after release.
⊙——⊙ 3-hour clamp. ×——× 5-hour clamp.

normal. In the 5-hour group there was considerable variation between animals killed at the same time after release of the clamp; thus in more than half the animals the pyro-P value was not significantly above the minimal ischaemic level, but reached 70 per cent of the normal value in other animals.

Adenosine-equivalent.—The variation in the adenosine-equivalent was of the same order as that of the pyro-P (Table II). It is of special interest that there was a positive correlation, illustrated in Fig. 1, between the pyro-P and the adenosine-equivalent of each sample of muscle, irrespective of the time after release at which the animal was killed, i.e. there was no accumulation of adenylic acid which remained un-phosphorylated.

Resynthesis of A.T.P.—It seems reasonable to conclude from these results, in agreement with Bollmann and Flock, that when the circulation is re-established after a moderate degree of ischaemia there is a resynthesis of A.T.P. in the muscle, but after prolonged ischaemia there is little or no resynthesis. The fact that the adenosine-equivalent and the pyro-P levels were parallel to each other suggests that the failure to resynthesize A.T.P. is not due to failure of the mechanism

rephosphorylating adenylic acid, but to a failure to reaminate inosinic acid. It is clear that if resynthesis of A.T.P. takes place the process is very rapid, but that there is a threshold, determined by the duration and degree of anoxaemia, beyond which resynthesis does not take place when the circulation is re-established. In our experiments the duration of ischaemia necessary to reach this threshold was approximately 5 hours, but the variation within this group, due presumably to variation in the fine adjustment of the clamp with a consequent variation in the degree of anoxaemia, indicates the sharpness of the threshold.

Changes in other constituents.—After 3 hours' ischaemia the water and chloride content of the muscle increased considerably within ½ hour after release of the clamp, remaining then more or less constant for the next 24 hours (Table II); the total P, pentose and K values were unchanged, showing that there had been no

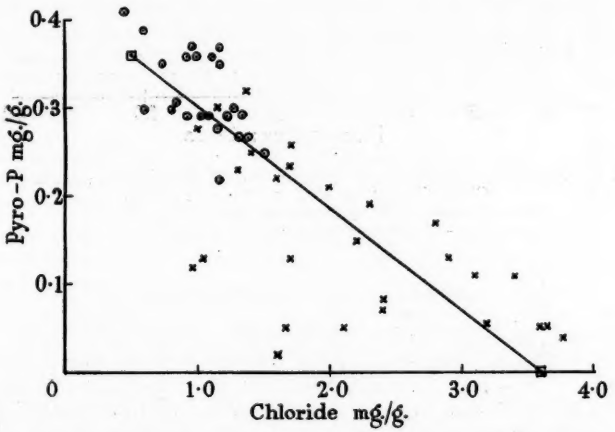

FIG. 2. Negative correlation between adenosinetriphosphate and chloride after release.
⊙——⊙ 3-hour clamp. ×——× 5-hour clamp.

significant leakage from the muscle. After 5 hours' ischaemia there was a rapid initial rise in H_2O and Cl, without appreciable loss of phosphate, but though the H_2O content then remained about the same level, the Cl content was still further increased in the later samples. Concurrently the total P, pentose and K content decreased and the Na value rose further.

Correlation between the pyro-P and Cl content.—Fig. 2 illustrates the relationship between the pyro-P and Cl content of the samples. When the pyro-P content was 50 per cent or more of the normal value (0·36 mg./g.) there was a marked negative correlation between the pyro-P and the Cl content of each sample, the points lying about a line drawn between the points for normal muscle and for plasma respectively. Below this level of pyro-P there is divergence from this line, the divergent points being from samples from animals in the 5-hour group killed soon after release of the clamp. The figures were therefore regrouped according to whether the pyro-P value of the sample was above or below 50 per cent of the normal value, and for each group the mean Cl content of

the samples taken at a given time plotted against this time (Fig. 3). At the higher pyro-P values, i.e. at the higher metabolic level, the maximum Cl content was 1·2 mg./g. muscle, and the level apparently was tending to return to normal 48 hours after release of the clamp ; but at the lower metabolic level, with little resynthesis of A.T.P., the initial rise in Cl was twice as great and the content ultimately approached the value for plasma Cl, indicating a change in the permeability of these muscles which was irreversible at least in the 48-hour period of these experiments.

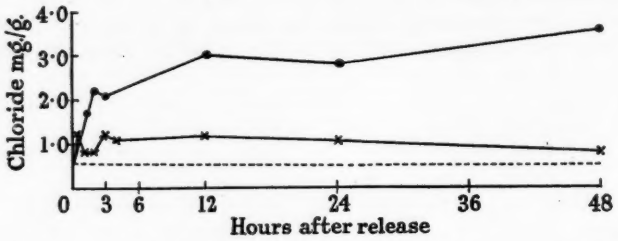

FIG. 3.—Time curve for chloride in samples with high and low pyro-phosphate.
⊙——⊙ Pyro-phosphate below 0·2 mg./g.
×——× Pyro-phosphate above 0·2 mg./g.
– – – – Normal chloride.

EXPERIMENTS WITH RABBITS.

The foregoing experiments with guinea-pigs confirm and extend the observations of Bollmann and Flock (1944) with rats, in showing that during ischaemia the A.T.P. of muscle is not only dephosphorylated, but also deaminated, and that failure to resynthesize A.T.P. on re-establishment of the circulation is accompanied by failure to reaminate the inosinic acid residue. These observations, however, are at first sight at variance with the results obtained by Billings and Magraith (1937), who found, in investigating the chemical changes in the tissues of rabbits after obstruction of the blood supply, that after tying the external iliac artery on one side, the adenosine-equivalent and the histamine content of the occluded limb muscle were considerably *increased* above the normal value. We have made similar experiments with guinea-pigs, tying the artery and vein, and were unable to detect any difference in these respects between the normal and the occluded limb, but in view of the findings of Griffiths (1940), it seems unlikely that the procedure results in an effective anoxaemia of the limb. It is not clear whether the results obtained by Billings and Magraith could be due to a change in the water content of the muscle.

Since it seemed of some interest to establish whether the changes in rabbit muscle during anoxaemia were similar to those of rat and guinea-pig muscle, we carried out in four rabbits, under nembutal anaesthesia, the procedure used by Le Gros Clark and Blomfield (1945) in their studies on the regeneration of muscle. The anterior tibial muscle on one side was carefully freed from its bed, and all visible vessels entering or leaving it were tied and cut, leaving the muscle attached only at its origin and insertion ; brom-phenol blue was injected intra-

venously 5 hours later, about 10 minutes before the animal was killed. In all the animals the dye had penetrated (as these authors also found) into the nominally ischaemic muscle, though the colour was somewhat obscured, particularly in the distal third, by the congestion of the vessels. Analysis showed that the water and chloride contents of the ischaemic muscle were considerably greater, but the pyro-P and adenosine-equivalent only slightly (13 per cent) less than the contents of the anterior tibial muscle on the normal side. In one rabbit, however, a small slip of the external longissimus muscle, the vessel to which had been presumably cut by accident during the operation, was clearly unstained, and its pyro-P content had fallen to 45 per cent of the normal value.

In another rabbit six turns of rubber tubing were applied as a tourniquet to the right thigh according to the method of Bywaters and Popjak (1942), with the difference that the first turn was made just *below* the knee-joint. After 5 hours the dye was injected and the animal killed; the muscles of the right leg from 2–3 cm. above the knee-joint downwards were unstained, and in these (anterior tibial and gastrocnemius) the pyro-P and adenosine equivalent had fallen to 40 per cent of the normal value. The thigh muscles underlying the tourniquet however were deeply stained, although the skin had been corrugated by the tubing.

It appears, therefore, that when the devascularization of rabbit muscle is complete as judged by the failure to stain with injected brom-phenol blue, the decomposition of A.T.P. proceeds in a similar manner to that of guinea-pig and rat muscle, but that in the rabbit the blood supply through the bony attachments may be sufficient to maintain the resynthesis of A.T.P. in the muscle for at least a considerable period (5 hours) of nominal ischaemia. It should be pointed out that our animals were not strictly comparable with those of Le Gros Clark and Blomfield, being quiescent after anaesthesia and confined in a small cage, while their animals had greater freedom for exercise, which would increase the oxygen demand; moreover, these authors noted that the devascularization was more obvious after 24 hours. Nevertheless it would be of interest to know whether the remarkable capacity for regeneration of the muscle fibres after necrosis in the circumstances which they described is associated with a survival of the metabolic capacity, and whether such regeneration would take place on establishment of the collateral circulation after complete arterial, as well as venous, occlusion.

DISCUSSION.

Green (1943) suggested that shock developing after release of a tourniquet might be due to the absorption of A.T.P. from damaged muscle. The fact that during the period of ischaemia the A.T.P. of muscle is decomposed makes it unlikely, as already pointed out by Bollmann and Flock, that this is a correct explanation; nor is there any evidence that if A.T.P. is resynthesized to an appreciable extent on re-establishment of the circulation there is any subsequent leakage of pentose-containing esters; there is undoubtedly a leakage of phosphate and of pentose compounds from muscles with a low pyro-phosphate content, but no evidence of any leakage of A.T.P. as such.

It appears probable that any relationship between A.T.P. and the onset of shock is due to the importance of this coenzyme in the normal metabolic cycle rather than to any toxic effect. Parnas (1929) showed that ammonia was formed

when muscles contracted in oxygen, and came to the conclusion that this ammonia was derived from adenylic acid, and that an oxidative reamination of inosinic acid took place in the physiological cycle. The identity of the NH_2-donator and the mechanism of the reaction are still unknown. Our experiments show that during ischaemia the A.T.P. in the occluded muscle is dephosphorylated and deaminated, and that after prolonged ischaemia reamination does not take place when the circulation is re-established.

It seems clear that this failure must in any case be of considerable consequence in the functional recovery of the muscle. Is it also a causal factor in the onset of shock? There appears to be a relationship between the degree of resynthesis of A.T.P. and those factors which are accepted as probable or possible direct causes of shock—the loss of water and other substances into the tissue and the absorption of toxic substances from it. When resynthesis of A.T.P. takes place to an appreciable degree, i.e. at least 50 per cent of the normal level, the entry of water and of sodium chloride into the muscle ceases a few minutes after the re-establishment of the circulation, and there is no appreciable loss of phosphate or potassium (and presumably no loss of less readily diffusible substances, such as enzymes) from the muscle. The amount of Cl entering appears to be inversely proportional to the amount of A.T.P. resynthesized. When, however, resynthesis of A.T.P. does not take place, there is a greater initial loss of water and sodium chloride into the tissue, and a further loss as the exudate forms and the Cl of the muscle comes into equilibrium with the plasma Cl; concurrently phosphate, potassium and no doubt other substances pass out from the muscle.

It may be noted that the loss of water and of Cl from the circulation takes place in two stages, and that in the second stage the loss of Cl is probably relatively more marked than the loss of water, for the water content of the muscle remains constant, while the Cl increases to the plasma level; both water and Cl are lost in the exudate. If the onset of shock is determined by the loss of a critical amount of water, or more particularly of NaCl, from the circulation, it is clear from Fig. 3 that both the quantity of muscle injured and the degree of injury determine whether this threshold is reached, and the time after release of the tourniquet at which it is reached. Thus, approximately twice the amount of muscle which must be occluded to produce *immediate* shock after 5 hours' ischaemia (Cl entry 2 mg./g. muscle) would have to be occluded to produce shock after 3 hours' ischaemia (Cl entry 1 mg./g.); but if a smaller amount of muscle had been occluded for 5 hours, shock might supervene 12 hours or so after release of the tourniquet, as the Cl content of the muscle rose to 3 mg./g. In the latter case the re-application of a tourniquet would prevent further critical loss; it has been shown by Shipley, Meyer and McShan (1945) that in their experimental conditions such re-application saved life.

Though it has not been shown that there is a causal relationship between the resynthesis of A.T.P. and the entry of water, chloride, etc., into the muscle, the facts described lend colour to the hypothesis that the normal permeability of the tissue is maintained by the metabolism of carbohydrate, and that the direct causes of shock may be referred to the failure of the metabolic cycle. If this is the case, as the metabolic level (indicated by the level of A.T.P.) falls during ischaemia, the permeability of the sarcolemma will increase; as soon as the circulation is re-established Na and Cl ions will pass from the extracellular fluid into the fibre, while the osmotic equilibrium which has been disturbed by

the accumulation of lactate, inorganic phosphate, etc., is re-established by the entry of water. If, with the re-establishment of oxygenation, resynthesis of A.T.P. takes place and the tissue rapidly returns to its normal metabolic level, the normal permeability will be re-established and no further entry of Na and Cl will take place. If, however, the mechanism of reamination has failed, the permeability will remain abnormal; the content of Na and Cl will increase, that of phosphate and K decrease, until ionic equilibrium with the plasma is established.

The reamination mechanism is perhaps of peculiar interest in that its failure as a result of anoxaemia may be a primary cause of tourniquet shock; it is possible that a relative failure of reamination in tissue anoxia due to haemorrhage might be a factor in haemorrhagic shock also. But it is obvious that a failure in the resynthesis of A.T.P. is not the only point at which the metabolic cycle could fail, for a similar effect would be produced by the heat-inactivation of the enzymes concerned or by specific enzyme poisons. Moreover an increase in the permeability of the cell membrane might be brought about by agents which directly affect the structure of the membrane (e.g. a lipolytic agent, such as the lecithinase present in *Cl. welchii* toxin) with the same overall "shock-like" effect. Aub, Bines, Kety, Nathanson, Nutt, Pope and Zamecnik (1945) have indeed suggested that the formation of clostridial toxins is a potent factor in the development of traumatic shock; this generalization is based on experimental work with dogs, which apparently harbour *Cl. welchii* in their muscles, and there is little indication that it is applicable to man or to other species of animals. On the other hand, field investigations and statistical studies on clinical gas gangrene indicate that in addition to any direct action of the bacterial toxins on vital organs, the presence of necrotic muscle in the body is a factor in the severity of the disease (McLennan and Macfarlane, 1945; Macfarlane, 1945); presumably whether such muscle is necrotic as a result of bacterial action or of ischaemia or trauma, it will act as a trap for water, salts and plasma, and a source of any autogenous toxin, so long as it is in effective connection with the circulation.

SUMMARY.

The pyro-P content and the adenosine-equivalent of muscle in the guinea-pig or the rabbit decrease during a period of ischaemia, indicating that A.T.P. is dephosphorylated and deaminated.

On re-establishment of the circulation after a moderate period of ischaemia, A.T.P. is resynthesized, but after longer occlusion there is no resynthesis. The failure appears to be due to the failure of the mechanism by which inosinic acid is reaminated.

There is a relationship between the level to which resynthesis of A.T.P. takes place and the rate and extent of entry of water, Na and Cl into the muscle and of the loss of other substances from the muscle.

The results suggest that the maintenance of the cycle for the metabolism of carbohydrate is a crucial factor in maintaining the normal permeability of the muscle fibre, and that the changes leading to clinical shock after the release of a tourniquet may arise from the failure to re-establish this cycle.

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